Loss of translation elongation factor eEF1A2 differentially affects pathways responsible for dying-back neuropathy and Wallerian degeneration in vivo

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The Summer Meeting of the Anatomical Society took place on 2–4 July 2008 at the new Jubilee Campus, University of Nottingham, UK.

S1. Implantation: deciphering the molecular cross talk

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Deciphering the molecular code that culminates in implantation is one of the most interesting challenges in human biology. Our group has approached this problem from the perspective of trophoblast differentiation. In this context, it is likely that a subset of the mechanisms that enable placental cytotrophoblast invasion of the decidua/myometrium during later pregnancy function during initial attachment of the embryo to the receptive uterus. We also reasoned that this process might involve molecules that mediate adhesion under conditions of shear stress in other locations. Using this combined approach we discovered a role for several families of molecules. For example, we showed that uterine epithelial expression of the unusual sulfated carbohydrate structures that mediate leukocyte rolling on endothelium, the first step in immune cell extravasation, is highly upregulated during the period of uterine receptivity. On the embryonic side, trophoblast cells that form the outer surface of the blastocyst express L-selectin, a carbohydrate-binding adhesion molecule that interacts with these specialized oligosaccharides. The net result of this multivalent, low-affinity interaction is a rolling motion, which we think mediates the initial relatively unstable step in embryo attachment, termed apposition. We envision that this receptor–ligand interaction produces signals that reverberate through a myriad of pathways including the chemokine and integrin systems that mediate leukocyte migration and stabilize adhesion, respectively. We also think that the field of human embryonic stem cell research will be an important source of information about the factors that govern differentiation of the blastocyst trophectoderm into adhesion-competent cells. Initial morphological studies from our group suggest that the cells of the human embryo have greater diversity than their counterparts in the mouse. The implications of this finding in terms of the molecular signals that mediate implantation are an open question that we are actively investigating. Our ultimate goal is to improve the success rates of assisted reproductive technologies by understanding the molecular underpinnings of the component processes, which involve both embryonic and maternal cells and the important dialogue that takes place between them.

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S2. Implantation *in vitro* and *in silico*

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The molecular mechanisms underlying hormonal control of human endometrial receptivity to the implanting embryo remain largely unknown. We have developed a model in which mouse embryos attach to human Ishikawa cells, which express functional progesterone and estrogen receptors. Blastocysts flushed from day 4 pregnant mice have high attachment potential on tissue culture plastic (~90%), but only ~40% progress to adhere stably to confluent Ishikawa monolayers. However, after 24 h, E_2 priming followed by 72 h incubation with medroxyprogesterone acetate and estradiol, stable adhesion increases to ~70%. Specific changes in glycosylation of cell surface mucin are seen in response to steroidal stimulation, both in the cell culture model and the epithelium of cycling endometrium. An informatics filter of transcriptomic data focusing on glyco-genes identified 140 candidates expressed in both Ishikawa cells and normal mid-secretory endometrium, of which 19 are integral to the plasma membrane (including several adhesion molecules), 64 are glycosyl transferases, 12 are calcium-related and 5 are lectins or lectin-related. One of these is a glycosyl transferase with a role in cycle-dependent mucin glycosylation. To target candidate mediators of early phase adhesion, apical epithelial glycoproteins were affinity-selected and then subjected to a proteomic protocol, leading to the identification of ~30 species, half previously unrecognized. Several candidates so far tested immunochemically are present at the apical surface of normal endometrial luminal epithelium. The *in vitro* model will allow their function in implantation to be explored.

S3. Trophoblast invasion and remodelling of the uterine vasculature: what comes first and what happens if both fail?

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At the time of implantation, spiral arteries within the maternal uterus have already been undergoing morphological changes long before any extravillous trophoblast cell has reached their wall or lumen. Only during week 5 p.m. of gestation do extravillous trophoblast cells first come into contact with the maternal decidua and start to invade this tissue. Following the interstitial route of trophoblast invasion, some of the extravillous trophoblast cells take a side route and transform spiral arteries into uteroplacental arteries. But, even if the erosion of such vessels begins about mid

first trimester, only at the end of the first trimester is a continuous flow of maternal blood into the intervillous space of the placenta established. The next weeks of pregnancy are characterized by a further widening and deeper erosion of the spiral arteries down to the first third of the myometrium. Also, at the periphery of the placenta, additional arteries are transformed to guarantee an adequate increase in blood supply to the placenta to cope with the growing needs of the fetus.

If trophoblast invasion and transformation of spiral arteries fail, the fetus needs to counteract this failure, and this becomes obvious also at the level of the placenta. As long as the blood transfer from the placenta to the fetus is not impaired, the placenta adapts to the new situation and starts to increase its surface by an increased branching angiogenesis, which leads to an increased number of terminal villi. This adaptation may not be sufficient and the fetus may subsequently run into an arrest or reduction of growth leading to intrauterine growth retardation (IUGR).

Based on the timing of trophoblast invasion and onset of maternal blood flow towards the placenta, it becomes obvious that IUGR cannot be based on an insufficient blood flow before the second trimester. New predictive markers for preeclampsia are significantly different already at 7 weeks of gestation, making it tempting to speculate that preeclampsia is not caused by a failure in trophoblast invasion.

S4. Trophoblast-mediated spiral artery remodelling: a role for apoptosis

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Trophoblasts (TC) detach from the anchoring placental villi and invade the uterine wall (interstitial invasion) and its blood vessels (endovascular invasion) as far as the inner myometrial segments. Trophoblast invasion occurs during the first 20 weeks of pregnancy; during this period TC, endothelial cells (EC) and vascular smooth muscle cells (VSMC) transiently coexist in partially modified spiral arteries. Access to maternal arteries by TC may be by endoluminal upstream migration or by trans-stromal migration and subsequent penetration of the arterial wall. As pregnancy progresses, TC surround and invade the artery walls, fibrinoid matrix accumulates and musculo-elastic tissue is lost. Immunohistochemical studies provide limited insight into cellular dynamics and molecular mechanisms, and are complicated by variations in the patterns of TC invasion and in artery modification in different areas of the placental bed. As a result, there have been disparate views regarding the role of TC in arterial remodelling. Although it has been suggested that some changes in the vessels are independent of TC, occurring as part of the maternal response to pregnancy, there is also evidence that invasive interstitial TC prepare the decidual spiral arteries for endovascular TC migration. What is clear is that in the absence of trophoblastic invasion the extent of this vessel remodelling is radically curtailed, indicating that trophoblasts must play an active role. In pregnancies complicated by pre-eclampsia, where invasion is reduced, remodelling is impaired. A number of mechanisms may be responsible for normal remodelling of spiral arteries including migration, de-differentiation loss of adhesion and apoptosis. In recent years we have investigated the role that apoptotic mechanisms may play. Trophoblasts synthesize a number of death-inducing ligands including tumour necrosis factor (TNF), FasL and TNF-related apoptosis-inducing ligand (TRAIL), which are either released or expressed on the cell surface. Using *in vitro* and *ex vivo* models developed in our laboratories we have investigated the interactions between trophoblasts and vascular cells. We have found that trophoblasts induce apoptosis in both EC and VSMC by both soluble and cell-associated mechanisms and we have implicated Fas/FasL and TRAIL/TRAIL-R pathways in this process.

S5. Cellular and molecular mechanisms that establish the feto-maternal interface in mice

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The mouse has become a commonly used model for understanding the development and biology of the placenta, taking advantage of the ability to make transgenic mutant mice, culture trophoblast stem cells, analyse nutrient transport capacity, and the availability of dozens of cell subtype specific markers. We now have molecular insights into the development of all trophoblast cell types in the placenta: trophoblast stem cells, spongiotrophoblasts, glycogen trophoblast cells, and parietal- and spiral artery-trophoblast giant cells (TGC) in the junctional zone; as well as two layers of syncytiotrophoblasts ands canal- and sinusoidal-TGC in the labyrinth. Growth of the placenta continues to near term but is driven by trophoblast cell hyperplasia only up to around mid-gestation (E12.5) and in later gestation by cell hypertrophy. The labyrinth contains highly branched villi and is the site of nutrient exchange between mother and fetus. The villi are covered by three trophoblast cell types that separate the maternal blood sinusoids from fetal capillaries – a single mononuclear cell that is a subtype of trophoblast giant cell (sinusoidal TGC) with endocrine function and two multi-nucleated syncytiotrophoblast layers, each resulting from cell–cell fusion, that function in nutrient transport. Our recent evidence indicates that the three differentiated trophoblast cell types in the labyrinth arise from distinct and autonomous precursors in the chorion that are patterned well before morphogenesis begins. Different TGC subtypes line the maternal blood spaces in the junctional and labyrinth zones. They are all post-mitotic and polyploid, and dependent on the *Hand1* transcription factor gene for their development, but they express different types of hormone genes, implying that they have unique endocrine functions.

S6. Oxygen, the Janus gas: its effects on placental development and function

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Life evolved in an anaerobic environment. The accumulation of oxygen in the atmosphere approximately 2 billion years ago presented opportunities for more efficient energy production, but

also posed challenges by exposing biological molecules to oxidative attack. Antioxidant defences developed to combat this threat, but nonetheless oxygen concentrations must be carefully regulated at the cellular level to avoid excessive oxidative stress. Recent research has demonstrated that during the first trimester of pregnancy the human feto-placental unit develops in a low oxygen environment. This may protect the embryo from oxygen free radical-mediated teratogenesis during the critical phase of organogenesis. During this period maternal blood flow to the placenta is severely restricted, and the feto-placental unit is supported by secretions from endometrial glands. Onset of the maternal arterial blood flow at the start of the second trimester causes a three-fold increase in intra-placental oxygen concentration, posing an oxidative challenge to placental tissues. Therefore, onset must be carefully co-ordinated, and in normal pregnancies is a progressive phenomenon, starting in the periphery of the placenta. Here, locally elevated levels of oxidative stress lead to increased apoptosis and regression of the villi over the superficial pole of the chorionic sac, forming the chorion laeve. In pathological pregnancies, onset is both premature and disorganized, secondary to deficient trophoblast invasion. Overwhelming oxidative damage to villous tissues contributes to loss of these pregnancies. Deficient trophoblast invasion is associated with incomplete conversion of the maternal spiral arteries, increasing the risk of spontaneous vasoconstriction. We have proposed that, in later pregnancy, this can cause repetitive low-grade ischaemia-reperfusion-type injury to the placenta. Manipulations of placental explants *in vitro* demonstrate that hypoxia–reoxygenation creates the oxidative stress and changes in gene transcripts and pro-inflammatory cytokines that typify the placenta in preeclampsia. Similar changes are seen *in vivo* in normal placentas subjected to labour compared to caesarean-delivered controls. Fluctuations in oxygenation are also a powerful stimulus of endoplasmic reticulum stress. Our most recent data indicate that this may suppress activity in the AKT/mTOR pathway, leading to impoverished placental development in cases of intrauterine growth restriction. *Supported by the Wellcome Trust.*

S7. Nutritional programming of disease: unravelling the mechanism

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Nutritional programming is the process through which variation in the quality or quantity of nutrients consumed during pregnance exerts permanent effects upon the developing fetus. Programming of fetal development is considered to be an important risk factor for non-communicable diseases of adulthood, including coronary heart disease and other disorders related to insulin resistance. Early studies of the nutritional programming concept relied upon epidemiological approaches and were subject to robust criticism on the grounds that such studies, often looking back over a period of 40–60 years, had failed to adequately adjust for confounding factors. Animal models of programming have been developed, which have utilized restriction or over-feeding of specific nutrients in either rodents or sheep. These consistently demonstrate the biological plausibility of the nutritional programming hypothesis and, importantly, provide tools with which to examine the mechanisms through which programming may occur. Studies of animals subject to undernutrition *in utero* generally exhibit changes in the structure of key organs such as the kidney, heart and brain. These appear consistent with remodelling of development, associated with disruption of cellular proliferation and differentiation. Whilst the causal pathways which extend from this tissue remodelling to disease can be easily understood, for example a kidney with fewer nephrons has reduced function, ultimately driving development of hypertension, the processes which lead to this disordered organ development are poorly defined. A simple explanation might be that maternal undernutrition leads directly to fetal undernutrition and altered development, but this is challenging to determine. The placenta is a key controller of substrate availability to the fetus, and there is evidence that undernutrition causes vascular dysfunction in the placenta. Moreover, the placental regulation of materno-fetal endocrine exchange is also perturbed by nutrient restriction. Even minor variation in maternal nutritional status is capable of producing important shifts in the fetal environment. It is suggested that these environmental changes are associated with altered expression of key genes, which are responsible for driving the tissue remodelling response and future disease risk.

S8. Early embryo adaptations to maternal protein undernutrition and their consequences

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Periconceptional nutrition in diverse animal models has been shown to influence the plasticity of the developmental programme leading to altered postnatal phenotype affecting growth, physiological and metabolic parameters, often associated with onset of adult disease. We have investigated developmental programming mechanisms using rodent models in which maternal low protein diet has been administered for either the period of oocyte maturation before mating or preimplantation development after mating, with normal control nutrition provided for the remainder of gestation and postnatally. These models have demonstrated cardiovascular dysfunction, notably relative hypertension and associated disturbance in arterial vessel relaxation potential, behavioural abnormalities, metabolic effects and altered growth in offspring following periconceptional maternal protein restriction, with evidence of gender-specific sensitivities. Analysis of pre-implantation embryos derived from diet-challenged mothers indicates programming of developmental plasticity has occurred by the blastocyst stage. Our data indicate that the extra-embryonic lineages associated with both the development and function of the visceral yolk sac and trophoblast are altered by the diet challenge suggestive of compensatory mechanisms to increase the efficiency of nutrient retrieval likely to protect fetal growth and stabilize competitive fitness in the next generation. Activation of these adaptive responses and resulting changes in fetal development appear to involve physiological and epigenetic components. Collectively, our rodent models provide evidence to support the concept that the developmental origins of adult disorders can be traced back to gamete and embryo environmental quality. *Funded by NICHD, USA and MRC, UK.*

S9. The placental insulin-IGF-system in diabetic pregnancies

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Owing to its two separate surfaces, the placenta is exposed to regulatory molecules such as growth factors and cytokines in the maternal and fetal blood streams. Insulin (I) and insulin-like-growth factors (IGF)-I and -II have received particular attention because of their well-established mitogenic potency and their altered levels in conditions associated with fetal/placental overgrowth such as in (pre)gestational diabetes. Moreover, their receptors (R) are expressed in the placenta.

IR location varies with gestational age. Early in gestation, IR are predominantly expressed on the syncytiotrophoblast facing the maternal circulation. At the end of gestation, the majority of receptors are located on the placental endothelium and, hence, are in contact with the fetal circulation. This developmental change in receptor location is paralleled by a change in receptor activation, but not in intrinsic activity, resulting in changes in global as well as in specific gene expression.

The IR exist in two isoforms that differ by inclusion (Ex11+) or exclusion (Ex11–) of exon 11. Isoform expression differs between trophoblast (preponderance of Ex11+) and endothelium (more Ex11–). As Ex11– is a signalling receptor for IGF-II, its endothelial location enables fetal IGF-II to regulate as yet unknown functions in the placenta. Placental overweight in Beckwith-Wiedemann syndrome, which is characterized by a duplication of the fetal IGF-II gene, suggests placental endothelial cell growth as one endpoint controlled by fetal IGF-II in addition to insulin.

Early in gestation, maternal insulin can stimulate placental growth via activation of the MAPK pathway and modify placental development by inducing placental matrix-metalloprotease 14, which is upregulated in Type-I diabetic pregnancies. At the end of gestation, fetal insulin will activate metabolic pathways in the endothelium via protein kinase B including endothelial accumulation of glycogen, a well-known feature of the placenta in diabetes. Diabetes is also accompanied by a change in IR splicing favouring the long IR isoform that mediates mitogenic effects. This will further enhance the growth-promoting role of insulin on the placenta.

IGF-IR are predominately located on the basal membrane of the syncytiotrophoblast, thus allowing access of fetal IGF-I to the receptor. This strongly argues also for a fetal-placental IGF-I axis that may be modified in diabetes, including stimulating placental amino acid transport to help sustain fetal growth.

S10. Vascular dysfunction in the diabetic placenta: causes and consequences

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Implantation, placentation and embryonic/fetal development are vulnerable to the maternal environment. The maternal diabetic milieu in pregnancies complicated by Type-1, Type-2 or gestational diabetes is pro-inflammatory, where increased growth factors, glucose, insulin and tissue hypoxia all can contribute to vascular disturbances in the placenta. *Ex vivo* placental perfusion studies, whole tissue explant cultures and endothelial cells *in vitro* show that the vascular system therein responds by altering to a pro-angiogenic and pro-permeability phenotype. Augmented transplacental glucose flux and the resultant fetal hyperinsulaemia appear to induce also changes in the fetal blood vessels of the placental barrier. The ratio of the pro-angiogenic and anti-angiogenic growth factors are altered in diabetic pregnancies, as is junctional maturity and vascular permeability. The increased chorionic villous arborization and angiogenesis seen in diabetic pregnancies would also affect placental maternal blood flow and nutrient uptake. All these changes can impact on fetal growth and well being, given the major contribution of the placental endothelium to transplacental resistance and materno-fetal transport. Indeed the phenotypic changes in the diabetic placental vasculature may extend upstream to the fetal vascular system. Certainly, we have evidence of changes in the umbilical vein endothelial cells (HUVEC) taken from diabetic pregnancies; cells here show hypertrophy and increased sensitivity to VEGF, which is retained for several passages, suggesting epigenetic alterations may have occured during gestation in a diabetic environment. These studies strengthen the importance of the intra-uterine environment in dictating placental and fetal vascular function. *Funded by The Wellcome Trust, ASGBI, MRC.*

S11. A stereological perspective on placental morphology in normal and complicated pregnancies

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Stereology provides sampling and estimation procedures for quantifying the 3D morphology of biological structures (organelles, cells, tissues, organs, organisms) from physical, optical and other slice images. Its practical utility is explained by the fact that global quantities (total volumes, surface areas, lengths, numbers), average values (e.g. mean particle size) and tissue distances (e.g. membrane thickness) can be obtained with minimal bias and high precision. Over the past 20 years, technical developments have improved these properties and formalized optimal methods. Here and elsewhere, researchers have applied stereological principles to human and animal placentas. As a result, it is now possible to quantify relevant processes (including villous growth and development, trophoblast turnover, fetoplacental angiogenesis and diffusive transport) and to test whether these are compromised in pregnancy complications. Studies on global quantities show that villous maturation normally involves differential growth of fetal capillaries driven primarily by increases in endothelial cell number. Angiogenesis is biphasic with marked changes in vascular content and arrangement occurring around mid-gestation. Studying numbers of nuclei has shown that human trophoblast is a continuously renewing epithelium with a steady state between cytotrophoblast (CT) proliferation and recruitment and syncytiotrophoblast (ST) differentiation and extrusion. This produces a numerical ratio of ST : CT nuclei of about 10 : 1 whilst trophoblast gradually becomes

thinner because expansion of surface area exceeds growth in volume. Interestingly, the integration of all these growth processes results in changes in total diffusing capacity which match the growth in fetal mass. Not surprisingly, at least some of these processes are perturbed in complicated pregnancies. Recently, we have found that (1) fetoplacental vascular growth is compromised in pregnancies accompanied by maternal asthma, (2) alterations in trophoblast turnover differ between pre-eclampsia and intrauterine growth restriction, and (3) maternal vascular development is impoverished, but diffusive transport increases, in placentas from pregnant rats exposed to particulate urban air pollution. Innovations in quantitative immunoelectron microscopy have shown how stereology can reveal non-random distributions of proteins in different cellular compartments and whether spatial distributions alter following experimental manipulation.

Oral presentations – Session 1

Effects of hyperglycaemia and hypoxia on vascular endothelial growth factor expression in human chorionic villous explants

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The maternal diabetic milieu includes periodic hyperglycaemia and chronic hypoxia. Whilst vascular dysfunction is known to be a feature of the diabetic placenta, the direct effect of these on vascular development and function in the human placenta is not known. The aim of this study was to look at the effects of hyperglycaemia and hypoxia on vascular endothelial growth factor (VEGF), the key regulator of placenta angiogenesis and vascular permeability. A chorionic villous explant culture system was used as this allows long-duration culture, maintains 3D architecture and allows cross talk between the endothelium and adjacent trophoblast. Explants were taken from normal term placenta ($n = 3$) obtained by elective Caesarean section. They were incubated for 24 h in Medium 199 (with 5% fetal calf serum and 100 U mL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin) at 37 °C, with or without the addition of 10 mM glucose (to give a final concentration of 15 mM glucose) and hypoxic insult (1% oxygen). Mannitol 10 mM was used as the osmotic control for the high glucose cohort. In the glucose studies, 50% of blood vessels in the explants showed VEGF immunoreactivity in the fetal endothelium. A contingency table analysis (Chi-squared) revealed a significant difference ($P < 0.001$) in level of VEGF immunoreactivity between 5 mM and 15 mM glucose samples at 24 h, which was in part (but not wholly) caused by the higher osmolarity. In the hypoxic samples, there was a significant increase of percentage of vascular profiles positive for VEGF ($P < 0.05$; Student's t-test) and in secreted VEGF levels in the supernatant (ELISA). In explants from both high glucose and hypoxia studies, changes in VEGF were found only in the vascular endothelium, not in the overlying trophoblast layer. Our data suggest that the human placental vascular bed is vulnerable to increased glucose and hypoxia, two of the main characteristics of diabetic pregnancies. *Funded by ASGBI.*

In vitro dual perfusion of the human placental lobule as a phantom to investigate the relationship between fetoplacental flow and 3D power Doppler signal

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Abnormalities in placentation and impaired placental circulation can lead to fetal growth restriction. We aimed to test the hypothesis that increased fetoplacental blood flow correlates with increased power Doppler vascular indices (VI, FI and VFI) using the *in vitro* dual perfusion model of the human placental lobule.

Three term placental lobules were dually perfused through both circulations with Earle's bicarbonate buffer (EBB), gassed with 95% O_2/5% CO_2. The fetal side was supplemented with diluted heparinized fetal blood (as a Doppler scatterer) and flow adjusted from 1–10 mL min^{-1}; maternal-side flow was maintained at 14 mL min^{-1}. Prior to commencing the experiment, fetal resistance was stable at < 70 mmHg and venous outflow at > 80%. Images were obtained with a Voluson i (GE Medical Systems) USS and a neonatal transducer. Three 3D datasets were acquired at each flow rate from each placental lobule and these were measured in triplicate using VOCALTM. A sphere was centred on the cotyledon along the chorionic–decidual axis, at a constant site, with a diameter corresponding to placental thickness. Written consent was obtained from each subject.

Mean VI showed a high degree of correlation ($R^2 = 0.911$, $R^2 = 0.946$, $R^2 = 0.962$) with total fetal-side flow for each lobule. Similar results were seen for VFI. The relationship between FI and flow was more complex and non-linear. Spearman's correlation coefficient was statistically significant ($P < 0.001$) for data analysis and image acquisition, confirming reproducibility. Increasing depth between the placental tissue and the probe, by addition of tissue mimic blocks, caused a significant reduction in signal. No signal was seen at a depth of 6 cm or greater.

There is a positive linear correlation between fetal flow and power Doppler signal but it is markedly affected by attenuation. These data provide qualifying information for clinical translation, where gestational fetoplacental blood flow will be assessed to attempt the prediction of fetal growth restriction.

A role for angiostatin in the pathophysiology of fetal growth restriction?

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Activation of inappropriate haemostasis in the placenta and fibrin deposition are associated with pregnancy complications and generation of a range of pro/anti-angiogenic proteins at the cell surface. Levels of free plasminogen, a key haemostatic cascade

pro-enzyme that is pro-angiogenic, increase in normal pregnancy and may contribute to the growth and maintenance of the vasculature. Angiostatin$_{4.5}$ ($AS_{4.5}$, an anti-angiogenic 52-kDa proteolytic cleavage product of plasminogen) is produced at the cell surface by tethering of plasminogen to β-actin and its subsequent cleavage by uPAR. We hypothesized that AS is capable of inducing human FGR and that the mechanism is related to trophoblast turnover and generation of AS on the villous surface. *In vitro* studies reveal a dose-dependent effect of $AS_{4.5}$ on apoptosis and the rate of wound healing in human trophoblast cell lines (SGHPL-4 and HTR8/SVneo), with no such effect in non-trophoblast cell lines. Preliminary results of dual immunostaining have revealed an increased level of co-immunoreactivity for β-actin and uPAR within human fetal growth restriction (FGR) samples (36.19 μm^3 vs. 15.39 μm^3 in control placenta) which was significant at a *P* value of 0.05. The proportion of cells which are immunoreactive for cleaved Caspase-3 (an effector caspase in apoptosis) is increased within human FGR placentas (25.65 μm^3 vs. 8.66 μm^3 in control placenta). These results provide evidence that angiostatins are implicated in the pathogenesis of this condition.
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Placental renin expression in twin–twin transfusion syndrome and *in vitro* regulation of renin in placental cells

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In pregnancies complicated by twin–twin transfusion syndrome (TTTS) there is an imbalance in placental vascular connections in identical monochorionic twins resulting in net transfer of blood from one twin (donor) to the other (recipient). The donor has diminished blood volume, and the recipient an excessive blood volume, explaining many, but not all, of their phenotypic features. In this light we have recently shown that both twins show activation of the renin-angiotensin system (RAS). Activation of the RAS can be easily explained by the volume loss in the donor, but not in the recipient. We investigated the contribution of placental renin in TTTS tissues and placental explants. Ethics Committee approval was given for these studies, and all patients gave informed consent. First, we show in the recipient that expression of mRNA for renin, and renin protein is up-regulated in the placenta, as opposed to the kidney. Because hypoxia is one of the main upregulators of renin expression, we undertook *in vitro* studies in term placental explants. Low levels of oxygen (3% oxygen tension) increased renin protein, compared with placental normoxia (8%). Hyperoxia (20% oxygen) also increased renin in cultured explants, suggesting that stress may affect placental renin. Studies of the activity of renin promoter transfected into BeWo choriocarcinoma cells confirmed that 3% oxygen upregulated promoter activity, compared with 8% and 20% oxygen tension. These data show that placental renin expression is regulated by local oxygen tension, but the application of these findings to the altered placental renin levels in the recipient twin in TTTS is not straightforward. Placental oxygen tension is determined primarily by maternal oxygen supply, and there is no evidence that the donor and recipient sections of a TTTS placenta are differentially supplied with maternal blood. Further work is needed to identify the regulators of placental renin in these complicated pregnancies.

Blood flow and nutrient transport in the human placentone: a mathematical model

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Mathematical formulation of the human placental circulation and metabolic exchange was started more than 30 years ago by Longo et al. [L.D. Longo, H. Bartels (eds), *Nat. Inst. Child Health Human Dev*., 1972]. Nevertheless, it is still not clear how placental structure and geometry, in particular decidual vasculature distribution, affects intrauterine haemodynamics and solute concentration patterns, which could result in placental insufficiency. The aims of this study were to examine the influence of the relative positions of decidual vessels and maternal blood flow rates in a single placentone on the effectiveness of nutrient uptake, where the placentone is defined as a placental circulatory unit formed by a single villous tree and its corresponding maternal vessels. Our model considers steady filtration of an incompressible viscous liquid in a porous medium, and assumes that advective transport of solutes dominates molecular diffusion. Blood flow velocity and pressure fields are calculated analytically using the theory of images in a hemispherical domain. The concentration field is described by a solute transport equation incorporating different types of uptake kinetics. We investigated the dependence of net nutrient uptake rate on the system parameters. We showed that a ratio of solute consumption rate to maternal blood flow rate imposes a restriction on homogeneous spatial solute distribution. The model suggests that a large distance between the decidual artery and veins provides more effective metabolic exchange, supporting the hypothesis of basal veins' location on the periphery of the placentone.*Supported by Marie Curie EST Network MMBNOTT.*

Oral presentations – Session 2 (ASGBI-funded Student presentations)

Molecular basis of digit identity in embryonic limb development

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A fundamental problem in biology is how structures form in the correct place in developing embryos. We are interested in limb development and, specifically, how anatomically distinct digits form at different positions along the anteroposterior axis of the limb. *Sonic hedgehog* (Shh) is known to be pivotal in determining the number and pattern of digits but little is known about genes that are expressed in response to Shh, and how these genes are translated into digit anatomy. We have taken two approaches to identify genes that could be involved in determining digit identity in the limb: one in which we use *Drosophila* wing vein patterning as a model, and the other, microarrays. In *Drosophila*, genes of the *Iroquois (Irx)* complex contribute to the specification of individual wing veins. We have compared *Irx* gene expression in the developing

limb/fin of mouse, chick, human and zebrafish. This analysis reveals both similarities and differences in the expression of these genes. We have also tested whether *Irx* genes might mediate digit development by experimental manipulations in chick embryos and have shown that expression is regulated by Shh and BMPs. The microarray has uncovered genes that are expressed in specific digit primordial. This should help to define the complete digit patterning process.

Conditional immunoablation of NG2-glia to study their functions

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NG2-glia are an abundant population of glial cells in the CNS identified by their expression of the surface antigen marker NG2 proteoglycan. NG2-glia generate oligodendrocytes during development and after demyelinating injuries in the adult CNS. However, NG2-glia are also widely distributed in non-myelinating regions and their complex morphology, characterized by highly branched processes, suggests that they might represent a novel specialized population of glia. Notably, they contact nodes of Ranvier in the white matter and form synapses with neurones in the gray matter, where they are able to respond to neuronal activity. It has been suggested that NG2-glia are involved in the neuronal–glial signalling by modulating synaptic transmission, but current approaches have not clearly addressed these questions. For example, NG2 knock-out mice failed to reveal major defects and targeting the cells themselves may be more relevant for studying their functions. Here, we describe the development of an immunotoxin system for the conditional ablation of NG2-glia. We first tested the immunotoxin *in vitro* to prove its efficacy in ablating NG2-expressing cells. A control NG2-negative cell line was also used to assess its selectivity. The *in vitro* results indicated that the immunotoxin was both effective and selective in inducing cell death in the NG2-expressing cells. We then examined the ability of the immunotoxin in ablating NG2-glial cells *in situ*, using organotypic cultures of brain slices. The immunotoxin was also effective in this *ex vivo* condition, as shown by post-treatment immunohistochemistry and live cell confocal imaging. The development of a specific system for the selective ablation of NG2-positive cells is an approach to investigate the function of this newly identified population of glial cells. Future *in vivo* studies will provide the possibility to shed more light on the functions of this abundant population of glia.

Gap junctions and hydrogen peroxide contribute to the EDHF response in omental veins from normal pregnant women

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Various substances are released from the vascular endothelium including nitric oxide (NO) and endothelium-dependent hyperpolarizing factor (EDHF). Although the latter factor has yet to be identified, several candidates have been suggested from studies on arteries. Candidates include potassium ions, hydrogen peroxide, epoxyeicosatrienoic acids and a role for gap junctions. Here, the endothelium-dependent vasodilators released from the omental veins from normal pregnant women, in response to bradykinin, were investigated.

Omental veins obtained from biopsy samples taken during elective Caesarean sections were isolated, cannulated and pressurized to 10 mmHg in a pressure myograph. After a period of equilibration, veins were preconstricted with U46619 until their internal diameter was 50% of the original. Responses to bradykinin (10^{-10} M–10^{-6} M) were determined and the presence of connexins (Cxs, the component proteins of gap junctions) was investigated by immunostaining using a connexin-specific 1° antibody, a biotinylated 2° antibody and a 3° streptavidin TRITC fluorescent antibody. The presence of the NO synthase inhibitor L-NAME (10^{-4} M) alone significantly reduced the vasodilator response to bradykinin, and the residual EDHF response was abolished in the presence of both L-NAME and KCl (45 mM). The EDHF response was significantly reduced by the gap junction un-coupler carbenoxolone (10^{-4} M), or after the decomposition of hydrogen peroxide with catalase (4000 units mL^{-1}). Cx37 was found to be present in the endothelium, Cx40 in the endothelium and smooth muscle, and Cx43 was present throughout the vessel wall

	Maximum relaxation % ± SEM			
Treatment	Control	Treatment	*n*	*P*-value
L-NAME	81.1 ± 4.0	60.8 ± 4.0	5	$P < 0.05$
KCl	81.8 ± 6.6	71.2 ± 11.6	5	ns
L-NAME + KCl	64.5 ± 7.1	16.7 ± 10.0	6	$P < 0.05$
Carbenoxolone*	47.6 ± 16.1	36.8 ± 6.9	5	$P < 0.05$
Catalase*	60.6 ± 6.7	41.6 ± 7.6	5	$P < 0.05$

*L-NAME (10^{-4} M) present throughout.

L-NAME and KCl abolished the vasodilator response to bradykinin demonstrating endothelium-dependent release of NO and EDHF in omental veins. The EDHF response was reduced in the presence of carbenoxolone or catalase, indicating that the bradykinin-induced EDHF response is composed of a hydrogen peroxide-induced vasodilation and a small role for gap junctions. The immunohistochemical location of connexins 37 and 40 in the endothelium suggests a possible role for gap junction communication between endothelium and smooth muscle cells.

Wnt signalling regulates transcription factor networks in vertebrate heart development

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The heart is the first functional organ in vertebrates but the mechanisms regulating its formation are not yet fully understood. One of the most important and elementary steps in this process is the decision of mesodermal cells to assume a cardiac fate and subsequently differentiate into heart muscle. This decision is regulated by cell-to-cell signalling including the Wnt family of secreted signalling proteins. In this study, we investigated the hierarchy of effects that β-catenin (an important regulator of canonical Wnt signalling) and GATA family members GATA6 and GATA4 have on heart formation in *Xenopus*. This was achieved using a combination of experiments utilizing inducible β-catenin and/or GATA mRNA

constructs and a potent pharmacological Wnt signalling agonist BIO. The effects of these manipulations on heart development were assessed by analysing gene expression of early and differentiated cardiac markers using RNA *in-situ* hybridization and quantitative polymerase chain reaction (QPCR). Further experiments have also been carried out to ascertain whether this regulation is cell autonomous or non-cell autonomous using the fluorescent lineage marker to map the migration of β-catenin throughout the embryo and to assess whether the loss of heart marker expression occurs in the same cells. Results show that overexpression of β-catenin leads to down-regulation of these heart markers, whereas GATA overexpression leads to an increase in marker expression. Overexpression of both β-catenin and GATA results in a return to normal levels of expression in these markers, suggesting that GATA activity is downstream of β-catenin and is a relevant target of Wnt/β-catenin in heart development. Experiments have also been carried out using an antisense morpholino strategy to target and knockdown XWnt6 expression alone, or in combination with BIO, to assess whether this Wnt ligand is a candidate for β-catenin-dependent endogenous regulation of *Xenopus* heart markers and/or heart formation. Further experiments are now being performed to assess if this regulation of GATA family members by β-catenin is direct or indirect. These experiments will make use of BIO and will incorporate cyclohexamide, a known inhibitor of protein synthesis.

Oral presentations – Session 3

The effect of Penitrem A on the developing neonatal rat cerebellum

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The neurotoxin Penitrem A has been shown in adult rats to specifically destroy cerebellar Purkinje cells (PCs) at narrowly defined concentrations mimicking closely the PC loss found in the cerebellar ataxias. The neonate rat cerebellum is relatively immature at birth, having a multicellular PC layer. Here, we report the effect of Penitrem A on this PC population. P3 (postnatal day 3, $n = 6$) neonates, P6 (postnatal day 6, $n = 6$) neonates and young adults (11 weeks, $n = 6$) received i.p. injections of Penitrem A (2–4 mg kg^{-1}). After periods of up to 72 h, the rats were killed by transcardiac perfusion with 4% PFA under halothane anaesthesia. PCs were identified either by Cresyl violet staining or by specific antibody to calbindin present on the PC surface. Wax sections (5 μm thick) were cut and the morphology and number of PCs were counted using the fractionator method. Data were analysed statistically and P-values < 0.05 were considered significant. Penitrem A administration caused noticeable tremor in all treated animals lasting for 1–3 days and was more immediate in the rat pups than in the 11-week-old rats. Quantification of Cresyl violet-stained sections showed that PCs were preferentially lost in the cerebellar vermis and specifically in folia VI–IX ($P < 0.001$~0.05) of P6 neonates 72 h after treatment. No change occurred in PC number in folia I–III and folium X. A 15% overall PC loss was observed in the P6 neonates with respect to the controls after 24 h, and 24% PC loss was seen after 72 h. However, there was no significant change in the PC number in P3 neonates. In 11-week-old rats, the pattern of PC loss was similar to that of P6 but the overall loss increased to about 49%. These results were confirmed by the loss of calbindin-binding cells in the PC layer and the appearance of enlarged vacuolated mitochondria. The results of this study show that Penitrem A can remove PCs in the immature rat cerebellum and thus provide a potential model to study the *in vivo* microenvironmental cues for the differentiation of PCs in the postnatal developing cerebellum and for stem cell therapy for trauma injury.

Ultrastructural organization of collagen fibrils in the dermis of α11-integrin deficient mice

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Integrins enable cells to interact with the surrounding extracellular matrix (ECM), thus providing attachment but also transmission of stimuli, which may influence cell behaviour. α11β1 integrins are major collagen receptors and are expressed in skin. Although skin tissue is rich in collagen fibrils, mice deficient for α11 integrin (one of the major collagen receptors) do not display obvious morphological alterations in the skin. However, MMP alterations in dental tissue occur. MMPs play a central role in remodelling ECM *in vivo*. We aimed to answer the question as to whether α11 integrins are able to control signalling pathways which can influence collagen fibril deposition and arrangement in the dermis. Therefore, the influence of α11 integrins in the deposition, aggregation and spatial arrangement of collagen fibrils in non-wounded skin tissue was examined in mice lacking α11 integrin. Tissue samples were taken from three wild-type (WT) and five alpha-11 knockout (KO) mice. Samples were fixed and processed for transmission electron microscopy. Ultrathin sections were cut and examined under a transmission electron microscope and the ultrastructural arrangement of collagen fibrils was evaluated stereologically. To this end, 10 images of non-overlapping regions of superficial dermis were taken adopting a systematic random sampling strategy from each mouse running parallel to the dermal–epidermal junction in correspondence with the interfollicular epidermis. Photographs were taken within a constant distance from the dermal epidermal junction. Quantitative analysis included the percentage of extracellular space (ECS) occupied by collagen fibrils, length density (Lv) of the collagen fibrils, the mean distance (mD) between fibrils and the size of the collagen fibrils. The data showed that there is no statistically significant alteration in the percentage of the ECS occupied by collagen fibrils, in their Lv or in the mD between fibrils due to the lack of α11 integrin. With regard to the size of collagen fibrils, KO and WT mice showed a quite uniform pattern of distribution of size of the fibrils, with the exception of one KO mouse in which the percentage of small fibrils within the 0–35.7-nm range was, on average, 30% higher than any of the other mice. The data suggest that tissue α11β1 integrins do not seem to have an essential influence on arrangement and aggregation of collagen fibrils in non-wounded skin connective tissue.

Inguinal herniae of the third kind – a new variant?

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Inguinal herniae are classified anatomically into indirect and direct types. Indirect herniae pass through the deep ring into the inguinal canal and direct herniae emerge through Hesselbach's triangle. Moreover, the deep ring is bounded by the transversalis fascial sling and the inferior epigastric vessels medially. Although not directly connected, the inferior epigastric (IE) vessels and transversalis fascial sling are intimately related in most patients such that it has been accepted that both of these structures form the lateral boundary of a direct hernia and the medial boundary of an indirect hernia. During the past 6 months, four patients have presented with symptoms of an inguinal hernia and have undergone a laparoscopic (posterior) inguinal hernia repair. At laparoscopy, once the peritoneal covering was removed it was observed that the inguinal herniae in these patients occurred between the IE vessels and the transversalis fascial sling of the deep ring. These herniae did not pass through Hesselbach's triangle and did not go through the deep ring into the anatomical inguinal canal. In all patients there was a recess lying between the IE vessels and the deep ring. There was a definite separation of the IE vessels and the transversalis fascia around the deep ring. Yet, not only was there a definite recess separating the IE vessels from the transversalis fascial sling but the peritoneal hernia sac actually protruded through the space. This unusual separation of the IE vessels and the deep ring has not been described in the literature thus far and this type of hernia may be a new variant of an inguinal hernia. Furthermore, because these herniae do not pass through the deep ring/inguinal canal or through Hesselbach's triangle, they cannot be defined as indirect or direct, respectively. Although a femoral hernia has been referred to as a third type of inguinal hernia, the herniae described herein are perhaps more entitled to be defined as inguinal herniae of the third kind.

Challenges and prospects of switching to digital online summative assessment in Anatomy using WebCT – A 5 year experience at Sultan Qaboos University

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Although online teaching is increasingly used in medical education generally and anatomy education in particular, there were few reports of summative examinations administered online. The aim of this project is to develop an online assessment system in Anatomy that offers advantages over our current practice of physically having spotters in the dissecting room or of involving living models for evaluating living anatomy knowledge. *WebCT*, a course-management system, offers, amongst other things, the functionality of administering examinations and could provide immediate analysis of the questions with regards to difficulty and discriminative indices. We have used this course-management system to administer summative examinations in Anatomy during the preclinical years in an integrated systems-based curriculum. Learning objects such as radiological images, pictures of prosected specimens, line drawings, and short video clips were acquired from various sources. The images and video clips were resized for optimal viewing on the computer screen and uploaded onto the learning management system. During the period under study, there was clear reduction of staff contact time with the added advantage of offering good quality assessment materials for question banking. However, unexpected computer crashes, possible malpractice during the examination and question bank security are real concerns. We have overcome some of these issues by developing JAVA-based applets that disable unauthorized access before and during the exam, as well as keeping a backup plan in case of a computer glitch. Although time-consuming at the beginning, once questions are uploaded, it makes their retrieval and administration very easy. In addition, there is an initial investment in the resources needed (computers, software licence, learning objects, training). Overall, this WebCT system offers distinct advantages with regards to question quality and reduction of staff contact time compared to the traditional spotter examination.

Students' perceptions and approaches to learning anatomy in a system-based course using prosection and dissection

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To examine students' perceptions and experiences of learning anatomy, an on-line Likert-style questionnaire was administered during 2006 to students on a Bachelor of Medicine 4- and 5-year programme (n = 256, 23.8%). Statistical analysis revealed that students predominantly felt that understanding anatomy and working with human cadaveric material were very important parts of becoming a doctor. Students reported that working on cadaveric specimens was an effective way of learning anatomy but also found the amount of anatomy they needed to learn daunting. Student responses were correlated with their approaches to learning (ASSIST) scores (Smith and Mathias, *Clinical Anatomy* **20**, 2007) using a Kruskal–Wallis test. Significant relationships between the approach to learning anatomy adopted and students' perception and experience of anatomy were found. A deep approach to learning anatomy was correlated with students who reported that the most effective way of learning anatomy in the dissecting room was to get their hands in and feel for structures ($P < 0.01$), used anatomical terms and language at clinical opportunities ($P < 0.01$) and frequently used their anatomy radiology knowledge at clinical opportunities ($P = 0.02$). A strategic approach to learning anatomy was characterized by students responding highly to using course books ($P = 0.05$), working in groups ($P = 0.01$), learning natural variation ($P = 0.03$), quickly using their knowledge ($P < 0.01$) and using surface anatomy ($P = 0.04$). A surface approach to learning anatomy was associated with students who found anatomy learning daunting ($P < 0.01$), did not see the point to it ($P = 0.04$), felt the teaching style did not suit them ($P < 0.01$), had a strong motivation to pass exams ($P < 0.01$), felt anatomy was memorization-based ($P = 0.04$), struggled to build on their previous learning ($P < 0.01$) and were not confident in their knowledge base ($P < 0.01$). These outcomes provide clear associations between students' perception of the anatomy learning environment, the approach adopted and the anatomy activities students engage in.

Methods used in anatomical education in UK: data from ASGBI questionnaire

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Responses to a questionnaire on anatomy teaching for medical students in UK and Ireland, designed during a Senior Visiting Fellowship (awarded to ARMC) were received from 74% of the 34 schools of medicine which deliver identifiable anatomy courses. We present here reported data concerning methods used in anatomical education, Not all respondents answered each query. Student-directed/centered learning featured strongly in 68% of schools, little in 20%; problem-based and scenario-based learning strongly in 28%, little in 68%; project-based learning/teaching strongly in 28%, *little* in 44%; methods designed to reflect the intellectual development of students as they advance in the course strongly in 16%, little in 40%. Handbooks to guide students through the course are produced by 84% (not in 12%); 96% of courses recommend textbooks. Multimedia materials are produced in 76–80%; commercial multimedia used in 80%; but 8% do not use multimedia and only 3 (12%) share multimedia with other institutions. The International Anatomical Terminology is considered useful in teaching (and used to a variable extent) in 68–80% (not in 20%), in students' learning in 52% (not in 32%), and considered irrelevant by 20%; 36% think it should replace traditional terms. Small group tutorials are used academically in 52%, for personal enhancement in 32%, for both in 16%; in 20% the content is determined by students; in 24% academic and personal tutorials are given by the same people. The number of students at a tutorial varies from 2 to 32; at a lecture from 50 to 400, at a dissection session from 50 to 300; at a dissection table from 4 to 14 with one member of staff dealing with 6–50 students. Teaching staff mobility is considered useful by 56% and to be encouraged by 72%. There are striking differences in the form and delivery of anatomical teaching among schools but, in all, there is concern that the present situation is less than satisfactory and in need of improvement.

Poster presentations – A. Theme: Reproductive Biology

Developmental toxicity of ethanol in chick heart micromass culture can be prevented by addition of vitamin C

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It is well known that alcohol consumption during pregnancy is associated with a wide spectrum of embryonic and fetal malformations in humans as well as in various animal species. The spectrum of effects, often called fetal alcohol syndrome, usually results in growth retardation of the embryo, craniofacial abnormalities and malformation of the central nervous system and cardiovascular system. The most common heart abnormalities include atrial and ventricular septal defects, stenosis of the pulmonary artery and tetralogy of Fallot. Certain experimental studies suggest that reactive oxygen species or free oxygen radicals are involved in ethanol-induced developmental toxicity, yet little is known about the underlying molecular and developmental mechanisms. Vitamin C (ascorbic acid) is a water-soluble vitamin with potent antioxidant properties against several oxygen free radicals. Many observational studies have shown protective effects of vitamin C on ethanol-induced growth retardation and central nervous system abnormalities. The aim of the study is to evaluate the adverse effects of ethanol on chick cardiomyocytes, and to examine the protective effects of vitamin C in reducing the ethanol-induced damage. Embryonic hearts were dissected from 5-day-old white Leghorn chick embryos and the cells were isolated and cultured in 24-well plates containing DMEM culture medium. Cells were exposed to 100 μL mL^{-1} of ethanol and 100 μM vitamin C. Endpoints for cellular differentiation were observational scores at 24, 48, and 144 h following explantation. Cell viability was established with resorufin and protein content of cells. Statistical analyses for results were via one-way ANOVA AND Kruskal–Wallis tests. Ethanol dramatically reduced cellular differentiation, cell viability ($P < 0.01$) and protein content ($P < 0.01$) at 100 μL mL^{-1}. However, 100 μM vitamin C when administered concurrently with ethanol could significantly improve cell viability ($P < 0.05$), protein content ($P < 0.05$) and beating cardiomyocyte foci such that the values were comparable with control cultures. Supplementation with multivitamins containing vitamin C during pregnancy may be a useful therapy to prevent defects in heart development that may be brought about by ethanol.

The Sd (a)-glycotope, recognized by the CT1 monoclonal antibody, is a surface-marker for mouse uterine natural killer cells

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Uterine natural killer (uNK) cells are important regulatory cells in pregnancy. The lectin *Dolichos biflorus agglutinin* (DBA) has been shown to be a specific surface marker for murine uNK cells. The lectin can also be used to isolate these cells. DBA binds to terminal N-acetylgalactosamine (GalNAc) residues in several constellations. The purpose of the present study was to specify this lectin binding site on murine uNK cells. Placenta of CD1 mice (days 8, 14 and 16 of gestation) were fixed in 4% paraformaldehyde and embedded in paraffin wax. Sections were stained with the monoclonal antibody CT1, which recognizes the Sd(a)-glycotope (NeuAcα2,3 [GalNAcβ1,4]Galβ1,4GlcNAc). Antibody binding was detected using peroxidase/DAB or in antibody (CT1)/lectin (DBA)-double staining by two-colour double fluorescence staining. The CT1-antibody binds to the surface, but not to granules, of mouse uterine NK cells. DBA staining was found in the surface and granules of uNK cells. These results indicate that the Sd(a) glycotope is present on the surface of murine uNK cells. The CT1 antibody is an IgM which was originally raised against a cytotoxic murine T-cell clone. It is found on intestinal intra-epithelial lymphocytes and fetal thymocytes but it is absent from lymph nodes and spleen. Therefore, these data suggest that the CT1-antibody, which recognizes a specific carbohydrate epitope, may be a useful and very selective marker for murine uNK cells. Moreover, further identification of this epitope during uNK ontogeny may shed light on important aspects of gestation physiology.

Impact of caffeine and ethanol, the most common human teratogens/embryotoxins, on embryonic cells cultured *in vitro*

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Congenital malformations are the leading cause of infant mortality but at present 60–70% of congenital malformations are of unknown aetiology. Maternal exposure to caffeine (psychoactive drug) or ethanol (binge drinking), two of the most commonly ingested human teratogenic molecules, has been associated with embryological malformations and developmental defects. The exact underlying mechanisms of teratogenesis are unclear. This study investigates the effects of these molecules on the chick heart micromass model (MM) and on the differentiation of contracting cardiomyocytes from D3 mouse embryonic stem cell test (EST) system. White Leghorn 5-day-old chick embryo hearts were dissected to produce a cardiomyocyte cell suspension in Dulbecco's Modified Eagle's Medium (DMEM). D3 mouse embryonic stem cells were also induced to form embryoid bodies (EBs) upon removal of LIF, using the hanging drop method, and subsequently grown in culture to form contracting cardiomyocytes spontaneously. Cell cultures were incubated at 37 °C in 5% CO_2 (v/v) and observations made for cellular differentiation at 24 h, 48 h and 144 h. Cell culture activity was assessed using the Resazurin reduction assay for determining the metabolic activity of cultures and total protein determined via the Kenacid blue assay. In the chick heart MM model, the protein levels were unaffected by any concentration of caffeine up to 2500 μM ($P > 0.05$). However, cell activity was significantly reduced at concentrations above and including 100 μM ($P < 0.01$), although beating (cellular differentiation) was not affected until a concentration of 1000 μM ($P < 0.05$) was administered. In D3 EST system, the protein content decreased at concentrations above 1000 μM. Cell activity was affected at concentrations above and including 400 μM ($P < 0.01$), but cell beating was only decreased at above and including 1000 μM ($P < 0.05$) caffeine. In the case of ethanol, there was no effect on protein levels at concentrations up to 33 μM ($P > 0.05$) in either system. Cell activity decreased at concentrations above and including 10 μM ($P < 0.05$) in the chick heart MM model and above 33 μM ($P < 0.05$) in D3 EST system. Cell beating was decreased at concentrations above and including 22 μM ($P < 0.05$) for the chick heart MM model, but only at 33 μM ($P < 0.05$) and above for the D3 EST system. The effect of these molecules on connexin expression in these cell types indicates that reduced gap junction activity may be involved in the action of these teratogens. These experiments provide evidence that the chick heart MM and the D3 ESC system may be useful *in vitro* methods for studies of perturbation of embryonic development.

Using a minigene assay to assess the function of a polymorphism associated with intra-uterine growth restriction

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Intra-uterine growth restriction (IUGR) ranks high among the most common and costly obstetrical conditions. IUGR predisposes to postnatal mortality and morbidity as well as a large range of adulthood diseases. Epidermal growth factor (EGF) is considered a potential regulatory factor for both trophoblast invasion and proliferation processes, which are consistent with proper placentation, fetal growth and well being. Previous studies in our laboratory showed that SNP c2566 G>A of EGF exon 14, which is a part of EGF pre-pro-protein, is associated with IUGR. Mutations within exons may cause aberrant splicing by disrupting exonic splicing enhancer (ESE) motifs. *In silico* analysis using the ESEfinder program identified a putative SF2/ASF protein binding site ESE sequence motif (GAGAGTA) within the c2566G allele with the potential to enhance proper splicing and inclusion of exon 14 in the mature mRNA. The c2566 G>A SNP (AAGAGTA) disrupts this site, potentially leading to loss of enhancer function with possible exon 14 skipping. Using a minigene technique, we examined the effect of the c2566G>A polymorphism on exon 14 splicing in PL4 (extravillous trophoblast) and HepG2 (hepatoma) cell lines. RT-PCR of the minigene constructs showed identical patterns of splicing for c2566G and c2566G>A alleles, with inclusion of exon 14. We conclude that the c2566 G>A polymorphism does not cause skipping of EGF exon 14 in these cell lines and this mechanism is therefore unlikely to explain the association between c2566G>A and IUGR.

The role of hypoxia on human placental angiogenesis: an immunocytochemical study of term placental villous explants

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Hypoxia is known to be an important stimulus for angiogenesis. It acts by stabilization and translocation of hypoxia-inducible factor (HIF) to the nucleus, with subsequent activation and transcription of angiogenic genes which includes vascular endothelial growth factor (VEGF). VEGF activates mitogen kinases and phosphorylates endothelial junctional adhesion molecules, resulting in endothelial proliferation and migration. Maternal hypoxia is thought to initiate placental vasculogenesis and angiogenesis in the first trimester, whilst fluctuations in maternal oxygen levels are thought to dominate vascular development throughout gestation. To our knowledge, there have been no experimental studies demonstrating these hypoxia-induced molecular mechanisms in intact human fetal vessels and this was therefore the aim of the study. Chorionic villous explants, which allow a more physiological multi-layered system, were taken immediately from three normal term placentas delivered by elective Caesarean section. After excision, they were incubated in M199 with added 5% FCS for 24 h in normoxia to allow for recovery from excision injury. Explants were then subjected to a further 24 h in normoxia or 1% hypoxia ($n = 6$ per condition). Explants were fixed, frozen in nitrogen-cooled isopentane, cryosectioned and subjected to immunocytochemistry, using monoclonal antibodies against HIF1-α, VEGF and vascular endothelial cadherin (VE-cadherin). Systematic random sampling of the immunostained vascular profiles, followed by statistical analysis using an unpaired *t*-test, revealed a significant increase in the percentage of nuclear profiles with HIF1-α ($P < 0.001$) and villous profiles with VEGF ($P < 0.001$) following hypoxia. Loss of junctional localization of endothelial VE-cadherin (from continuous to discontinuous staining) was also seen in the hypoxia group ($P < 0.006$). This study shows that fetoplacental vessels within excised chorionic villi of normal term human placenta can react to external hypoxia. The latter

causes upregulation of key signal transduction molecules involved in the first stages of angiogenesis. Our studies strengthen the hypothesis that hypoxia is a major regulator of placental angiogenesis.

The effect of fetal hyperinsulaemia on human placental vascular function: perfusion of fetal microvascular bed with high insulin results in increased vascular leakage and loss of junctional beta-catenin

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In diabetic pregnancies, fetal hyperinsulinaemia occurs as a result of increased insulin production by the fetal pancreas in response to augmented transplacental glucose flux. The effect of this on placental vasculature is not known. This study is the first to examine whether high insulin, administered from the fetal side, can cause alterations in junctional occupancy of the key adherens junction molecule, β-catenin and vascular leakage. Microvascular beds of normal human term placentae were perfused using an independent maternal and fetal dual perfusion method. Human recombinant insulin (25 mU L^{-1}, the median value of insulin in cord blood serum from Type 1 diabetic pregnancies at term) was added to the fetal circuit of the experimental group (n = 3), but not to control perfusions (n = 3). After 20 min perfusion, a 76 M_r dextran tracer (0.5 mg mL^{-1}) was introduced to the fetal circuit for a further 10 min. After fixation and processing, systematic random sampling was used to analyse the extent of endothelial junctional β-catenin and associated tracer leakage. The insulin-perfused group displayed a significant ($P < 0.05$) loss of junctional β-catenin, with 47% of vascular profiles exhibiting complete loss of β-catenin, in comparison with 8% in normal placentae. In the insulin-perfused placentae, the mean percentage of vessels with tracer leakage was 28%, significantly ($P < 0.05$) higher than the 9% seen in control perfusions. The data suggest that fetal hyperinsulinaemia may contribute to junctional disruption, increased placental vascular leakage and altered beta-catenin signalling. Beyond implications for placental barrier function, the results raise the possibility that high insulin may cause alterations in the vasculature of the fetus, with implied potentially long-lasting effects on the infant.

Quantitative analysis of human placental angiogenic growth factors from the second trimester of pregnancy

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Successful pregnancy requires the development of a complex fetoplacental vascular network which can respond to metabolic demands of the growing fetus and adapt accordingly. Fetoplacental vascular development may also be regulated by temporal changes in oxygen tensions within the maternal uteroplacental vasculature. Whilst there is information on changes in the first and last trimester of pregnancy, vascular changes in the second trimester remain unexplored. The aim of this work was to investigate the temporal expression of key hypoxia-sensitive angiogenic growth factors from 12 weeks onwards. Hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and erythropoietin (EPO) were localized in paraffin sections of human placenta from 12 to 40 weeks of pregnancy. Immunoprotein expression of Angiopoietin-1 (Ang1) and its receptor Tie-2 were also used as indicators of vascular maturation. Systematic random sampling was used to calculate the percentage of immunostained blood vessels, followed by statistical analyses using one-way ANOVA. At 12 weeks of gestation, 78% of fetoplacental blood vessels in stem, intermediate and terminal villi were positive for VEGF; this increased to 92% by 26 weeks but declined thereafter. A large proportion of blood vessels (85%) showed HIF-1α staining in the second trimester. After 27 weeks, the proportion declined until only about 40% of blood vessels showed HIF-1α. EPO expression did not parallel that of HIF-1α. Instead, the proportion of vessels with EPO increased after 27 weeks. The percentage of vessel profiles with Ang1 and Tie2 immunoreactivity showed a significant increase (from 35% to 85%) as pregnancy progressed, suggesting that vascular maturity occurs from the second trimester onwards with maximal expression towards term. In conclusion, the distributions of the HIF-1α and VEGF staining suggest that the hypoxia-driven period of angiogenesis and vascular remodelling might persist up to 26 weeks. The delayed increase in EPO staining is puzzling and possibly explained by its independence of HIF-1α or its roles in other processes including production of red blood cells. During gestation, angiogenic growth factors cooperate effectively to create, establish, mature and maintain the fetoplacental vasculature.

The interhaemal glycocode: a control point for reproductive efficiency and speciation

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Evidence collected over many years, using lectin histochemistry on plastic-embedded material, has led us to the proposition that the heavily glycosylated surfaces of an implanting blastocyst and receptive endometrium interact in a highly-controlled and specific manner during implantation. In species showing epitheliochorial placentation, this relationship is maintained throughout pregnancy. Examination of the fetomaternal interface in pig, horse, camel and alpaca reveals that each has its own particular pattern of glycan expression, or glycotype, on the interacting fetal and maternal surfaces, and that closely related species (such as horse and donkey, pig and peccary) have very similar glycotypes. We refer to such mutual compatibility as the glycocode. Based on recent lectin histochemical studies on interspecies hybrids between Old and New World camelids, the camel and llama, we suggest that successful interbreeding requires the trophoblast to bear glycans that are complementary to those of the maternal host. Due either to genetic recombination or environmental factors that modify glycosylation, hybrid embryos may show glycotypes that are not compatible with the uterine epithelium. We speculate that this may account for the high pregnancy failure rate of hybrids. Conversely, selection by the maternal host of embryos with modified glycotypes may result in increased efficiency of placentation. Selection pressure downstream could modify the maternal glycotype and eventually lead to the creation of new species.

Effect of gestational protein restriction on growth and β-catenin expression in the murine placenta

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Increasing evidence suggests that maternal undernutrition results in smaller offspring, affects fetal development and programmes cardiovascular diseases in later life. The effect of gestational nutrition on development and functioning of the placenta, and the mechanism involved, requires further investigation. During embryonic development, β-catenin is known to be a key regulator of proliferation and growth. Vulnerability of β-catenin signalling to nutritional changes is not known. The aim of this study was to ascertain if disturbed development of placental tissue and blood vessels were features in mice fed on a 9% casein (low protein, MLP) diet and whether this correlates with expression of β-catenin. MLP and control (18% casein) placentas were analysed at two distinct embryonic stages (E14.5, E18.5) in accordance with Home Office regulations. Systematic random sampling was used to analyse surface expression of β-catenin and stereological techniques were utilized to assess changes in the fractions of labyrinthine blood vessels and all other placental tissue types. Length, diameter and total volume of blood vessels were measured. Statistical significance was calculated using a linear mixed model to take into account the hierarchical nature of the data or ANOVA. Nutrient-restricted animals showed a 27% decrease in placental weight at E18.5 ($P < 0.02$) and in fetal weight at both E14.5 (about 17%) and E18.5 (13%, $P < 0.0001$ at both ages). Despite the overall decrease in placental size, only the chorionic fraction was decreased (five-fold) in the E14.5 MLP group ($P < 0.001$). Fetal and maternal blood vessel lengths were reduced by a quarter in the E18.5 MLP group ($P < 0.05$) in comparison with age-matched controls. Maternal weight and litter size remained unaffected. In both MLP groups, perturbation of β-catenin was observed with an overall reduction of staining in the labyrinthine layer, a higher percentage of vessels here showing weak or no junctional immunoreactivity. The reduction in fetal weight from the early embryonic time point, reduced placental weight and reduced length of labyrinthine fetal vessels suggest that gestational protein restriction impairs both placental and placental vascular growth and functional efficiency. The altered placental β-catenin may be part of the mechanism which leads to decreased growth.

Villous density in normal and growth-restricted pregnancies – a simplistic analysis

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Quantitative analysis of the different structures that contribute to the human placenta is a complex challenge. A number of computer-based analyses are becoming available, and one of the challenges is to incorporate all the cellular and tissue features that may influence placental function or pregnancy outcome into an automated analysis. In this study we attempted to address a simple hypothesis – that villous density would be lower in pregnancies affected by intrauterine growth restriction (IUGR) than in pregnancies with normally grown infants. Tissue sections were assessed by visual estimation of villous density, and also by manipulation of digital images of tissue sections. In brief, digital images (×50 magnification) were converted to ADOBE PHOTOSHOP files. Villous components were selected (lasso tool), and moved to minimize the space between villous cross-sections. A grid was then superimposed, and the area occupied by villi determined. Tissues were obtained from normal term pregnancies (with or without labour), and from preterm deliveries (with preterm labour, or after Caesarean section for pre-eclampsia). Local Ethics Committee approval was given for these studies, and all patients gave informed consent. Visual estimation and the digital assessment did not correlate well; visual estimation normally overestimated the villous area, so the remaining analysis was performed on the digitally based information. In normal term placentae the villous area was 44.7 ± 2.1% (mean ± SEM), and in normal preterm placentae (no IUGR) the villous area was 42.4 ± 3.8%. The corresponding data from pregnancies with IUGR were 29.7 ± 4.6% at term, and 28.3 ± 2.4% if preterm. There seem to be no differences related to gestational age in these samples, and IUGR is associated with a ~30% decrease in villous area which was statistically significant ($P < 0.05$ by ANOVA). This simplistic assessment cannot discriminate between cause and effect, but suggests that a loss of 30% of placental villous volume may be linked to IUGR. Further work is needed to assess the precision of these data.

Vertebral column patterning in the chick embryo

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The influence of the anterior–posterior (A–P) polarity of the somites on the patterning of the dorsal vertebral column and spinal peripheral nerves is well known. In the ventral vertebral column, however, the mechanisms underlying metameric segmentation of the vertebral bodies and intervertebral discs are still unclear, despite widespread acceptance of Remak's classical model of 'resegmentation'. Lineage analysis of quail-chick somite grafts is superficially consistent with resegmentation but does not address whether signalling external to somites is required for recombination of somite-halves. Moreover, in teleost fish, there is recent evidence that the notochord, and not somite polarity, is responsible for patterning the ventral vertebral column. There are indications in the older literature of a similar role for the notochord in amniote vertebrates, in particular the observation that excision of the notochord in the chick embryo results in morphologically unsegmented ventral vertebral cartilage. However, the notochord contributes cells to the intervertebral disc (nucleus pulposus) and it is possible that segmental patterning persists after notochordectomy at stages preceding the failure of discs to form. Our main goal, therefore, was to determine whether external signals are required for segmentation of the ventral sclerotome in amniote vertebrates. A marker for segmentation of the ventral sclerotome is the *Pax1* gene, which is expressed in the sclerotome-derived intervertebral disc anlagen. Accordingly, we have assayed for early sclerotome segmentation using *in situ* hybridization for *Pax1* after various surgical manipulations in early chick embryos. We find that *Pax1* expression remains segmented despite ablation of the notochord or neural tube, indicating that segmentation is intrinsic to the sclerotome and independent of axial structures. Moreover, unilateral reversal of the A–P polarity of the anterior presomitic mesoderm gives rise to *Pax1* stripes that misalign at the notochord,

excluding the operation of a midline signal that coordinates vertebral segmentation in amniotes. Detailed assessment of the spatio-temporal evolution of *Pax1* expression suggests a possible source of segmental signalling in the sclerotome, based on confrontation between groups of cells positioned at alternating A/P boundaries. Future work will also address whether extrinsic signals regulate vertebral column patterning at earlier developmental stages, when the A-P polarity of the presomitic mesoderm is undetermined.
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Cranial foramina development in the chick embryo

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Cranial foramina are tiny holes that allow the entry and exit of blood vessels and nerves through the skull. Malformations of cranial foramina in the embryo and also closure in adulthood can lead to blindness, deafness and facial paralysis as well as raised intracranial pressure, which can be fatal. The cellular and molecular mechanisms of cranial foramina development have never been investigated. We have examined the appearance of cranial foramina from day 4 to day 20 of development in the chick embryo, using alcian blue- and alizarin red-stained whole skeletal preparations and histological stained sections. This analysis has shown that overt cranial foramina appear at day 6 once cartilage has begun to differentiate. Furthermore, foramina reduce in size as development proceeds. Immunohistochemical analysis of cranial foramina in the chick embryo has revealed that all cranial foramina contain a nerve and a blood vessel. Additionally, we have identified a unique type of blood vessel, not immunoreactive to smooth muscle actin, which appears in some cranial foramina. We suggest these blood vessels are part of a glomus body contained within the cranial foramina. These blood vessels can be seen from day 4 of development and may prove useful in identifying regions where cranial foramina will form before morphologically overt foramina are seen. The role of nerves in the development of cranial foramina in the chick embryo is being investigated by removing developing cranial nerves before axon extension. We are focusing on ablation of hypoglossal nerves and bilateral enucleations (which result in loss of the optic nerves). The appearance of these hypoglossal and optic foramina are being examined in whole skeletal preparations and tissue sections. We are also examining the expression of early cartilage marker genes such as Sox9 and aggrecan in the mesenchyme around blood vessels, nerves and glomus bodies before morphologically distinct foramina are visible. These investigations will aid our understanding of whether cranial foramina can be identified as discrete regions where cartilage differentiation is inhibited from the time blood vessels and nerves invade head mesenchyme, or whether this process occurs subsequent to this.
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Expression of pleiotrophin and its receptors in human trophoblast

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Pleiotrophin (PTN) is a heparin-binding protein with multiple activities in cell growth, migration and differentiation. PTN expression in trophoblast is restricted to primates in which an endogenous retrovirus is inserted upstream of the first coding exon, generating a phylogenetically new trophoblast-specific promoter within the gene. It has been proposed that PTN expression may contribute to the invasive nature of human trophoblast. To further elucidate the possible functions of PTN in human trophoblast, we sought to determine the expression patterns of PTN and its receptors anaplastic lymphoma kinase (ALK), receptor protein tyrosine phosphatase beta/zeta (RPTPβ/ζ), and Syndecan-1 and Syndecan-3 (SDC1 and SDC3) in human first trimester and term placental samples obtained under local ethical committee approval. Using reverse transcription-polymerase chain reaction (RT-PCR), we observed exclusive expression of PTN from the retroviral promoter in trophoblast lineages (cell lines and primary tissues), whereas expression in maternal decidua was from the phylogenetically older promoter. Using immunohistochemistry/immunofluorescence (IHC/IF), we observed intense PTN staining in extravillous cytotrophoblast and on the basement membrane of villous cytotrophoblast. Cell columns and syncytial knots showed more diffuse but positive staining for PTN, whereas the syncytial microvilli showed intense staining. Consistent with the latter observation, increasing levels of PTN, as measured by ELISA, were found in maternal blood as pregnancy progressed. All of the PTN receptors examined were expressed in multiple cell types, with significant overlap with PTN staining. In particular, strong SDC1 expression overlaps with PTN on the syncytiotrophoblast microvillous membrane and, similar to PTN, SDC1 and ALK are strongly expressed in extravillous cytotrophoblast. SDC3 exhibited diffuse cytoplasmic staining in villous cytotrophoblast, and apparent staining of the cell membrane in extravillous trophoblast. Both RPTPβ/ζ and SDC3 exhibited strong cytoplasmic staining of the syncytium, but differed in that SDC3, but not RPTPβ/ζ, stained the microvillous membrane. RPTPβ/ζ staining was also evident in villous cytotrophoblast and extravillous cytotrophoblast and in the villous mesenchyme. We conclude that PTN and its receptors co-localize at functionally important sites in the human placenta, which has implications for understanding PTN function in human pregnancy.

Identification of a novel component of the renin-angiotensin system in human placenta

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Angiotensin 3–8 (Ang IV) mediates various effects in different tissues by binding to its specific receptor, AT4R. The active site of AT4R has been identified to be an insulin-regulated aminopeptidase (IRAP). At low concentrations, Ang IV is vasodilatory in a number of vascular beds and increases blood flow via a mechanism

mediated by the AT4R. To date, AT4R expression has not been investigated in the placenta, thus the current investigation was performed to determine whether this receptor was present in pregnancy.

The study had Hospital Ethical approval; written informed consent was obtained from all women. Placental samples were obtained from four normal (NP) and four cases of pre-eclampsia (PE) at delivery. Gestational ages were 39 and 38 weeks, respectively. Samples were taken from three areas: near cord, middle and outer edge of the placenta. Paraffin-embedded sections were immunostained for IRAP reactivity using a rabbit polyclonal antibody (a kind gift from Professor David James, Garvan Institute, Australia). Immunoreactivity of trophoblast and uterine cell populations was assessed using a semi-quantitative grading system: Grade 0 = no positive labelling, 1 = 1–25%, 2 = 26–49%, 3 = 50–74% and 4 = 75–100% of cells positively labelled. Data are expressed as mean score (± SEM).

AT4R immunostaining was predominant in the syncytiotrophoblast and Hofbauer cells of all placental villi examined. No differences in AT4R expression were found between the different placental locations examined. AT4R positivity was reduced in near cord placental samples from women who suffered PE (1.75 ± 0.25) compared to women who enjoyed normal pregnancy (3.25 ± 0.48; $P = 0.0044$).

AT4R plays a role in the vascular changes that occur in normal pregnancy and alterations to AT4R expression may have a role in the pathophysiology of PE. Reduced Ang IV/AT4R binding may cause increased placental vasoconstriction, resulting in increased ischemia/reperfusion. This, in turn, may stimulate xanthine oxidase, leading to increased superoxide production. Further work is needed to clearly define the role of this newly identified component of the renin-angiotensin system in normal pregnancy and PE.

A stereological study on the effects of the Mirena/Levonogestrel releasing device on the morphology of the human myometrium

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The Mirena or Levonorgestrel (LNG) Intrauterine System is an efficient contraceptive device which also brings about a profound reduction in menstrual blood loss. It is a recognized treatment for menorrhagia. In the present study, we have employed stereological methods, in combination with a structured light microscope system, to estimate aspects of myometrial morphology. The myometrium was examined in two groups of women: hysterectomy samples from women exposed to Mirena/LNG and a control group. This study was performed with informed consent and appropriate ethics committee approval. The tissue was fixed and processed for light microscopy. Sections were stained with Acridine orange.

The Volocity Grid confocal system was used to obtain Z-Stacks. The volume-weighted mean volume of the nuclei in the myometrium was estimated using the point-sampled intercept method. There was no overall difference in the volume-weighted nuclear volume between the two groups studied. However, the coefficient variation was greater in the treated group.

The biological relevance of these findings is still being explored, however, this simple study illustrates the usefulness of the Grid confocal system, which provides a cost-effective optical sectioning tool.

Expression of the BK_{Ca} channel in human chorionic plate arteries

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Following implantation the placenta rapidly develops into a highly perfused tissue; this is initially brought about through remodelling of the uterine vasculature. The resistance arteries of the human placenta are important in controlling placental perfusion through vasoconstriction and vasodilation. The nature of the specific ion channels involved in controlling vascular tone of chorionic plate arteries is not known, although a calcium-sensitive potassium channel (BK_{Ca}) plays a role in other vascular beds. We were interested to find out if there was a difference in the expression of BKca across the fetal and maternal interface of the placenta. Biopsies of the myometrium from non-labouring pregnant women and placenta were collected from patients undergoing elective caesarean section with written informed consent. Explants taken from the stem villous artery (SVA) and the third branch of the chorionic plate artery (CPA) were used to culture smooth muscle cells. Immunofluorescence methods were used to detect the expression of the BK_{Ca} channel as well as alpha actin as a smooth muscle cell marker. For Western blotting, tissue samples from the placenta and myometrium were homogenized on ice and separated by 10% SDS-PAGE to quantify expression of the BK_{Ca} alpha mono subunit ($n = 6$). Immunofluorescence for the BK_{Ca} channel was detected in both CPA and SVA ($n = 3$). This was supported by Western blot analysis for the BK_{Ca} channel in myometrium and placental resistance arteries and showed bands of approximately 124 kDa ($n = 6$). We have successfully shown that the BK_{Ca} is expressed in both the SVA and CPA smooth muscle cells. Given the range of signalling pathways that link the BK_{Ca} to vascular function, these findings suggest BK_{Ca} may have a role to play in regulating vasorelaxation of the resistance arteries. This implicates BK_{Ca} as a potential target for treating placental disorders such as pre-eclampsia (PET) in which the vascular arteries are poorly perfused and do not respond well to vasodilators.

Poster presentations – B. Theme: Neuroscience

Hypoxia induces rapid and selective injury to α-motor nerve terminals

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A growing body of evidence shows that presynaptic nerve terminals throughout the nervous system are vulnerable to a range of traumatic, toxic and disease-related neurodegenerative stimuli. Using a novel *ex vivo* model, we have recently shown that α-motor nerve terminals in several mouse muscles are highly susceptible to hypoxia-reperfusion injury. Hypoxia for 2 h (< 0.25% O_2) followed

by reperfusion for 2 h in lumbrical muscles triggered loss of neurofilament 168 kDa (NF) and synaptic vesicle 2 (SV2) protein immunoreactivity in about 83% ($n = 28$) of presynaptic α-motor nerve terminals. This insult did not appear to affect post-synaptic endplates or muscle fibres. We also established that this loss of α-motor nerve terminal morphology occurs by a mechanism distinct from Wallerian degeneration, as the *slow Wallerian degeneration* (*Wld*^{s}) gene did not protect nerve terminals from these pathological changes. We now show that 1A muscle spindle afferents and γ-motor terminals appear to be more resistant to hypoxia-reperfusion injury compared with α-motor nerve terminals. Furthermore, we now demonstrate that significant loss of α-motor nerve terminals in mouse lumbrical muscles occurs in response to hypoxia alone, without reperfusion. A time series analysis shows that loss of NF/SV2 immunoreactivity in α-motor nerve terminals first appears after a 1.5-h hypoxia insult and significant loss appears after 2 h, a 23% ($n = 20$) and 44% ($n = 20$) loss, respectively. Collectively, these data suggest that α-motor nerve terminals are highly and selectively vulnerable to hypoxic injury and that pathology is rapidly induced within 1.5 h of hypoxia alone. These findings may have clinical implications for the use of surgical tourniquets and in the aetiology of many neurodegenerative diseases where mechanisms relating to hypoxia and hypoxia-reperfusion injury have been implicated. *Funded by Anatomical Society of Great Britain and Ireland (BB, THG, SHP), Medical Research Scotland (THG) and the BBSRC (THG).*

Comparative analysis of early axon tracts in the embryonic vertebrate brain

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During early embryonic development, initial nerve connections in the vertebrate brain form an array of longitudinal tracts, transverse tracts and commissures. These tracts will act as a scaffold for later, follower axons that allow more complex connections to be set up in the brain. The early axon scaffold has been identified in a number of species, and many of the tracts appear remarkably conserved between all vertebrates analysed. However, a direct comparison of early tracts between different species is lacking, and many of the tracts are poorly characterized. Our aim is to provide a detailed reference for the early neurons and tracts in the main vertebrate model organisms. An initial goal is to identify an antibody that can be used in mouse, chick, *Xenopus*, zebrafish, and cat shark to visualize all neurons in the embryonic brain. We are currently testing pan-neural antibodies such as anti-neurofilament and anti-βIII tubulin. Starting with chick and *Xenopus* as examples of amniote and non-amniote species, these antibodies are being applied to establish a time series of initial tract formation. The pan-neural staining is combined with retrograde labelling using DiI to highlight specific tracts. The comparative description of early neurons and their tracts in the embryonic vertebrate brain will provide insight into the evolution of the embryonic brain architecture. In addition, it will allow mapping the expression patterns of developmental control genes onto the early axon scaffold. Finally, the detailed analysis of the early tracts is critical for the interpretation of early neuronal phenotypes in gain- and loss-of-function studies.

Analysis of cellular environment of spinal cord lesion following transplantation of neural stem cells into injured rat spinal cord

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Traumatic spinal cord injury leads to severe and permanent neurological deficits. Although no effective therapeutic option is currently available, recent studies have shown that cellular transplantation strategies hold promise to enhance functional recovery after spinal cord injury. Neural stem cells (NSCs) have been shown to differentiate into neurons and astrocytes *in vitro* and in adult brain. After spinal cord injury, it is thought NSCs may boost neural repair by encouraging host cell regeneration and promoting remyelination of axons. In this study, we examined the cellular environment of the lesioned spinal cord following transplantation of NSCs. Mouse NSCs were transplanted into female Sprague Dawley rat spinal cords 1 week following a moderate contusion injury at T9. Control animals received a vehicle injection. All NSC-treated animals were treated with Cyclosporin-A for the duration of the experiment. Animals were allowed 2 and 6 weeks' survival time post transplantation of NSCs. Spinal cords were fixed, frozen and sectioned. Immunohistochemical analysis determined the phenotype of cells surrounding the lesioned spinal cord and stereology was used to quantify these cells. Significantly fewer glial fibrillary acidic protein immunoreactive astrocytes and fractin immunoreactive apoptotic cells were observed at the lesion site in the NSC-treated animals. This is encouraging as astrocytes make up part of the inhibitory glial scar. The presence of NSCs may prevent some of the apoptosis that occurs after the inflammatory response to the injury. The beneficial effect of NSCs was further highlighted by increased presence of oligodendrocytes and less inflammatory microglia in NSC-treated animals. Stereological methods were used to estimate the volume of the lesion and volume of engrafted NSCs. Masson's trichrome staining revealed that most collagen staining was localized at the lesion site. Surface features of the spinal cords were analysed using scanning electron microscopy. Features of apoptotic cells were obvious, as were the NSC cell processes. Evidence of cell migration was found in scanning electron microscopy images. This analysis of the spinal cord lesion environment shows promising results for the potential use of NSCs in treatment of spinal cord injury.

Abnormalities of endoneurial vasculature in the peripheral nerves of genetically diabetic (db/db) mutant mice

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C57BL/Ks (db/db) inbred mice have an hereditary autosomal recessive disease resembling, in some respects, the human non-insulin dependent (type 2) diabetes mellitus. There is a reduction in myelinated fibre size in this mutant. Structural alterations in axonal cytoskeleton in unmyelinated axons in the tibial nerve of

these animals may provide a structural correlate of reported transport abnormalities. The present study is an electron microscopical, stereological study of the endoneurial vasculature. The tibial nerve of 9-month diabetic animals was compared with that from non-diabetic (m/m) controls (n = 5 in each group). This study had appropriate ethical committee approval. The parameters estimated were length density, and radial diffusion distance, endo-neurial area, perineurial area, endothelial cell and basal lamina thickness. We found that, in contrast to the control mice, there was a significant increase in the length density of endoneurial blood vessels and corresponding reduction in radial diffusion distance in the db/db mice. There were also slight (though not significant) alterations in perineurial and endothelial thickness and basal lamina thickness. These may indicate an adaptive response of the vasculature by attempting to reduce diffusion distance to overcome a potentially hypoxic environment.

Apolipoprotein E (APOE) genotype influences degenerative and regenerative events in the peripheral nervous system

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The Apolipoprotein E (APOE) gene encodes the major lipid transport protein in the nervous system. APOE is polymorphic, with ε2, ε3 and ε4 alleles giving rise to the three protein products: E2, E3 and E4 respectively. The APOE genotype is a major risk factor in CNS neurodegenerative disease, with the ε4 allele being associated with increased incidence of Alzheimer's disease. However, little is known about the effect of APOE genotype on degenerative and regenerative events in the peripheral nervous system (PNS). Here, we tested whether the APOE genotype influences the time course of degeneration and regeneration in the PNS after experimental nerve crush injury. Transgenic mice lacking APOE, or expressing human E3 and E4 on a null mouse APOE background, underwent a bilateral tibial nerve crush. Lumbrical muscles, immunocytochemically labelled to reveal neuromuscular junctions, were analysed 24 h, 2 weeks or 3 weeks later. Complete removal of APOE had no effect on degeneration or regeneration compared to wild-type muscles, confirming the results of previous studies. However, mice expressing the ε3 allele showed modestly delayed Wallerian degeneration with fewer vacant endplates and more endplates with associated presynaptic nerve fragments remaining than wild-type, knockout or APOE4 mice at 24 h post nerve crush. APOE3 mice also showed significantly accelerated initial regeneration of peripheral nerves at 2 weeks post crush ($P < 0.05$ compared to knockout; Kruskal–Wallis with Dunn's *post-hoc*), in agreement with studies in the CNS showing improved regenerative capacity. Conversely, the ε4 allele attenuated the re-innervation process, significantly delaying the re-establishment of normal mono-innervated neuromuscular junctions at both 2 and 3 weeks after nerve crush ($P < 0.05$ and $P < 0.001$ compared to knockout, respectively). We conclude that distinct APOE isoforms are capable of differentially modifying degenerative and regenerative processes in the mouse PNS in response to traumatic nerve injury.

Loss of translation elongation factor eEF1A2 differentially affects pathways responsible for dying-back neuropathy and Wallerian degeneration *in vivo*

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Mechanisms regulating neuronal viability are highly compartmentalized within neurons, with distal compartments – axons and synapses – being particularly vulnerable to a range of neurodegenerative stimuli. Numerous compartment-specific cellular pathways can bring about axonal and synaptic degeneration. For example, Wallerian degeneration, originally described as the response to traumatic nerve injury, is characterized by rapid, synchronous fragmentation (normally complete within 12–24 h) and organelle depletion. In contrast, dying-back neuropathies, commonly associated with conditions such as motor neuron disease, are characterized by a slower, progressive distal to proximal neuronal withdrawal in the presence of intact organelles. Despite distinct morphological characteristics, the convergent or divergent molecular mechanisms underlying these neurodegenerative pathways remain elusive. In this study, we examined neurodegenerative mechanisms in the naturally occurring '*wasted*' mutant mouse which carries a null mutation in the gene encoding elongation factor eEF1A2, an isoform of eEF1A. This mutation disrupts protein synthesis specifically in adult neurons and muscle, and has previously been shown to induce degeneration of lower motor neurons. Using high-resolution analysis of synaptic and axonal morphology we show that neurodegeneration in *Wasted* mice occurs by a 'dying-back' mechanism, morphologically distinct from Wallerian degeneration. We show that dying-back pathology in Wst mice cannot be accounted for by downstream reductions in levels of Smn protein (known to interact with eEF1A proteins and cause dying-back pathology in spinal uscular atrophy). However, levels of the zinc finger protein ZPR1 were modestly reduced to a similar level in Wst mice and $Smn^{-/-}$;SMN2 mice (a model of spinal muscular atrophy) suggesting that ZPR1 may play a common role in regulating dying-back pathways. Surprisingly, we also found that Wallerian degeneration was almost completely absent 24 h after an experimental nerve lesion in *Wasted* mice. This work demonstrates that Wallerian degeneration and dying-back neuropathy occur via divergent mechanisms *in vivo* with differing responses to disruption of eEF1A2 expression. Furthermore, eEF1A2-dependent molecular cascades are required for the normal initiation and progression of WD, supporting the hypothesis that WD is an active rather than a passive process.

Development of functional regionalization in the human neocortex: preliminary findings of SLIT/ROBO/srGAP differential expression

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Developmental lesions can cause significant rearrangement of functional regionalization of the cortex. Understanding the developmental mechanisms of cortical regionalization may make

it possible to harness the developing brain's inherent plasticity to overcome disabilities induced by pre- or perinatal brain damage. The extent to which regionalization is either genetically preprogrammed or created by epigenetic mechanisms, is still unknown. In rodents, certain transcription factors (e.g. PAX6, EMX2) are expressed in gradients across the cortex, independent of thalamic innervation. Perturbation of these gradients leads to altered regional expression of cell adhesion molecules and their receptors and to changes in organization of area-specific thalamocortical afferent projections. Our tissue *in situ* hybridization (TISH) studies and Affymetrix® gene chipping of transverse slices of human fetal brain (obtained from the MRC-Wellcome Trust Human Developmental Biology Resource, collected following British national guidelines with maternal written consent and ethical approval from the local Health Authority) have found that PAX6 and EMX2 are also expressed in counter gradients in the proliferative zones of the human neocortex at the onset of cortical plate formation. However, soon after and unlike the rodent brain, the PAX6 gradient disappears as EMX2 expression translocates to the cortical plate, whilst maintaining its gradient. Lists of genes with a potential rostro-caudal expression gradient have been obtained from 8–12 post-conceptional week (PCW) fetal brains by gene chipping. We have identified a number of cell adhesion and axonal guidance molecules expressed differentially across the rostro-caudal axis including members of SLIT/ROBO/srGAP family genes. SLIT and ROBO proteins are involved in axonal guidance and their absence leads to abnormal projection of various axon tracts (Bagri, *Neuron* **33**, 2002; Lopez-Bendito, *J. Neurosci.* **27**, 2007) In addition, they play roles in guiding cortical interneuron tangential migration (Andrews, *J. Anat*. **211**, 2007) and neuronal morphogenesis (Andrews, *Dev. Biol*. **313**, 2008) during rodent corticogenesis. Preliminary TISH and immunohistochemistry (IHC) studies have confirmed SLIT1, ROBO1 and its downstream signalling molecule srGAP1 display layer-specific and high rostral to low caudal expression profiles at 9PCW. We propose to investigate further the expression pattern of SLIT/ROBO/srGAP across different developmental stages, and aim to establish a linkage between their functions and regionalization of human neocortex.

Does cyclophosphamide cause chemobrain?

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After undergoing chemotherapeutical treatment for breast cancer, many patients report a reduction in their ability to concentrate and form new memories. This cognitive decline is colloquially termed 'chemobrain' and has been reported to continue even years after treatment has been completed. Adult neurogenesis is the proliferation of neuronal stem cells in the brain and its occurrence in the hippocampus has recently become associated with formation of new memories. There is evidence that cytotoxic drugs can target these new neurons, causing chemobrain. However, previous patient-based studies have been limited by many confounding factors and individual chemotherapeutical drugs need to be investigated. The present study looks at cyclophosphamide (CP), which is used as an adjuvant treatment for many cancers including breast. It is able to cross the blood–brain barrier and we wish to investigate its effect on both cognitive performance and hippocampal cellular changes. In accordance with Home Office regulations, Lister Hooded rats were randomly assigned to a drug-treated (n = 12) or vehicle group (n = 12) and administered (i.v.) with either a 30 mg kg^{-1} dose of CP or an equivalent volume of saline, with a total of eight injections over 3 weeks. Memory was tested 6 days after the final injection using the novel object location test. A Western blotting assay was used to quantify double cortin (a protein expressed in immature neurons) in the hippocampi and frontal cortices (a positive control) of the animals after death. The behavioural test showed the drug-treated animals to have no reduced ability to distinguish an object in a novel location from that in a familiar one, compared to saline-treated controls. No significant difference was found between quantities of double cortin in each group. The results indicate that CP did not cause any short-term effect on hippocampal-dependent memory formation and hippocampal neurogenesis. The longer-term effects of cyclophosphamide need to be investigated, along with more in-depth cellular analysis.

Sox gene expression in the adult mouse cerebellum

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Bergmann glia or Golgi epithelial cells are a discrete cell population located in the cerebellar cortex, where their radial processes extend from the Purkinje cell layer to the pial surface. Bergmann glia cells surround Purkinje cell bodies in the Purkinje cell layer and their radial processes are closely intertwined with Purkinje cell dendrites. Bergmann glia are known to play a crucial scaffolding role during cerebellar development, providing guidance both for Purkinje cell dendrites and for migratory granule cells from the external granule layer.

Although Bergmann glia are essential for the development and correct arborization of Purkinje cells, little is known about the regulation of this cell population post development. In an effort to characterize Bergmann glia at the molecular level, we analyse marker expression in these cells and compare it to other mouse brain populations. Using strategies including *in situ* hybridization and immunostaining, we demonstrate that mouse Bergmann glia co-express Sox1, Sox2 and Sox9, a feature otherwise associated with neural progenitors in the brain. In particular, Sox1/2 are typically involved in embryonic and adult neurogenesis and are considered to be reliable markers of neuroprogenitors in the CNS. Our results show that the Bergmann glia population displays specific expression of critical marker genes in the brain, suggesting a broader role for this cell population than previously thought. *J.A. was funded by a grant from the BRC (Univ. of Nottingham). V.S. is indebted to the Anne McLaren fellowship scheme (Univ. of Nottingham) and to the Alzheimer's Society for their support past and present.*

Bergmann glia of the early postnatal rat cerebellum express stem cell markers and retain neural stem cell characteristics in neurospheroid cultures of comparable ages

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Neural stem-like cells have been found in the postnatal and adult mouse cerebellum. This is indicative of a stem cell 'niche' that may prove important in cell replacement therapy. Here, we test whether this is true for the rat cerebellum and whether these cells can retain their neural stem cell characteristics *in vitro*. Newborn rat pups, postnatal days (PND) 3, 5, 7 and 16, were killed by an overdose of halothane followed by neck dislocation according to schedule 1 killing procedures. The cerebella were dissected out and fixed in 4% paraformaldehyde for 24 h at 4 °C and stored in 30% sucrose prior to cryostat sectioning at 12 μm. For spheroid culture, the cerebella of PND 1, 5, 7 and 10 were enzyme-dissociated and the cells seeded in stem cell-proliferating media for periods of up to 6 days. The spheroids were then fixed in 2% PFA at 4 °C and cryostat-sectioned at 12 μm. Sectioned material was incubated with primary antibodies to nestin, GFAP, Sox2, CD133, Ki67 and calbindin and visualized using indirect fluorescent secondary antibody techniques. At PND1 to PND10, nestin and GFAP were co-localized in the molecular layer in Bergmann-like radial fibres running perpendicular to the cerebellar surface from the superficial external germinal layer (EGL) to below the Purkinje cell layer (PCL). These cells had lost nestin staining by PND16 while retaining GFAP positivity. Sox2 staining was localized to the PCL. Ki67 was co-localized with nestin in the GL and highly proliferative EGL. At PND5 and PND7, CD133-positive cells showed increased specificity for the calbindin-positive PCL. PND10 and PND16 were negative for CD133. The cerebellar spheroids increased in size inversely with PND with a central core of GFAP staining cells surrounded by co-localized nestin/GFAP positive cells. The results show that a stem cell-like population, which appears to be Bergmann-like glia, exists in the postnatal rat cerebellum up to the third postnatal week and can be maintained in spheroid cultures of a comparable age. These data suggest that, in contrast to previous studies in mice, stem-like cells do not persist in the adult rat cerebellum, indicating possible species differences.

Effect of the chemotherapeutic agent, 5-fluorouracil, on memory and neurogenesis in the adult rat hippocampus

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Deteriorations in cognitive function have been reported by cancer patients undertaking chemotherapy. These include problems with memory and may indicate that chemotherapy directly affects the hippocampus. However, numerous confounding variables have made interpretation of patient studies difficult. This study is part of the development of an animal model to investigate the effects of chemotherapy on cognition and neurogenesis within the hippocampus. The dentate gyrus of the hippocampus is one of two brain regions to maintain adult neurogenesis. In most situations, changes in the formation of new neurons in the hippocampus correlate with changes in memory ability. Our hypothesis is that the cognitive deficits experienced by patients on chemotherapy result from a decrease in neurogenesis within the dentate gyrus.

To test this, the chemotherapeutic agent 5-flurouracil (5-FU) was administered to adult hooded Lister rats (dose 25 mg kg^{-1}). Cognitive behaviour was tested using the conditioned emotional response test (CER) and the object location test (OLR). In addition, the number of dividing cells in the subgranular zone of the dentate gyrus was quantified by staining for the cell proliferative marker (Ki67). Preliminary results (10 controls and 8 dru-treated rats) showed a significant decrease in the measured freezing time of the CER test in 5-FU-treated rats compared to controls and a nonsignificant decrease in the exploration time of objects in a new location. Also, the number of Ki67-positive cells in the 5-FU-treated group was significantly lower than in the control group. We are now looking for BDNF protein levels in both controls and drug-treated rat brains using the Western blot technique. From these findings, we conclude that 5-FU has an effect on hippocampus-dependent behaviour which is associated with a reduction in cell proliferation. This animal model confirms the reports of cognitive deficits made by patients taking this drug.

Effects of valproic acid on hippocampal-dependent cognition and proliferation

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The anti-epileptic drug valproic acid (VPA) is used extensively to prevent seizures but in addition this drug also acts as a histone deacetylase inhibitor and consequently reduces cell proliferation. Two regions within the adult mammalian brain, the olfactory bulb and the dentate gyrus of the hippocampus, continue to generate new neurons (adult neurogenesis) throughout life. These are generated by the proliferation of neural stem cells. The rate of adult neurogenesis in the hippocampus is correlated with the degree of hippocampal-dependent learning and memory. As there are reports of cognitive deficits by patients taking VPA, we suspect that this may be caused by the drug reducing adult hippocampal neurogenesis.

Adult Hooded Lister rats were given daily i.p. injections of VPA (300 mg kg^{-1}) for 10 days. Cognition was assessed by the object location recognition (OLR) and conditioned emotional response (CER) behavioural tests. These showed that animals treated with VPA performed significantly worse on the OLR test but showed no effect in the CER test. After behavioural tests, the animals were put down and half of each brain was used to determine the effect of VPA on cell proliferation within the subgranular zone (SGZ) of the dentate gyrus by immunostaining for Ki-67. This showed that VPA significantly reduced cell proliferation in the dentate gyrus of adult rats. The other halves of the brains were used to quantify DCX and BDNF proteins in the hippocampus by Western blotting. BDNF was significantly reduced in the hippocampus of VPA-treated animals compared to the controls. However, the levels of Double cortin showed no change.

This study shows that VPA reduces cell proliferation in the dentate gyrus and that this is associated with a significant effect on the ability of animals to perform a hippocampal-dependent behavioural test. The reduction in cell proliferation caused by VPA is associated with a reduction in the amount of BDNF but not Double cortin in the hippocampus. These results substantiate patient reports of cognitive impairment after administration of VPA and provide a mechanism by which it acts directly on neurogenesis within the hippocampus.

Poster presentations – C. Theme: Anatomy Education

Introducing anatomy learning packages into clinical attachments: the students' view

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In recent years, there has been increasing concern about the level of anatomical knowledge gained by medical undergraduates and retained into the later clinical years of the undergraduate training programme. In Aberdeen, we have resolved to address this issue, in part through developing a programme of learning packages designed to be delivered alongside attachments in the clinical phase (Phase III) of the curriculum where students rotate through all the specialties in a series of nine 5-week blocks which are delivered in an integrated systems-based fashion. We report on a study which sought the views of students on the need for such an initiative: from students at the beginning of their clinical attachment phase wherein they receive their core clinical knowledge training (Group A) and a second group (Group B) who had completed this phase and progressed into the fifth and final year (professional practice) of their undergraduate training. Students in both groups were asked closed-style questions in a written questionnaire during a time-protected slot on a training day. When asked: *Do you feel there is a need to develop anatomy teaching during Phase III?* – 95% (Group A) and 94% (Group B) said yes. *Do you feel there is a need to develop other basic science teaching during Phase III?* – 75% (Group A) and 57% (Group B) said yes. *What would be the best time to offer a teaching package*? – 87% (Group A) and 67% (Group B) said either before the start, or on the first day, of the clinical attachment. *How would you most prefer to complete a teaching package?* – 48% (Group A) and 53% (Group B) selected completing a teaching package by way of self-directed learning but in an environment with a staff member available. The majority of the remaining students (28% of Group A and 22% of Group B) indicated that they would prefer to complete a teaching package using a web-based online format. The results clearly indicate a desire amongst the student population for learning packages to provide vertical integration of anatomy, and also of the other basic sciences. Despite the seniority of these student groups, there was still a strong desire for staff support for such learning packages if they are to be included in the curriculum.

Introducing anatomy learning packages into clinical attachments: a way forward

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Past curricular changes in the Aberdeen medical undergraduate curriculum led to the restriction of formal anatomy teaching to the first two terms of the first year of the undergraduate medical curriculum. Over recent years, and not a new phenomenon, there have been consistent concerns about the level of anatomical knowledge and understanding that students have when they reach the wards and are receiving their clinical training. Whilst the reasons for these concerns are almost certainly multifactorial, and varied between individuals, there is nevertheless a need to address the issue and consider what can be done to improve the situation. We have developed a prototype learning strategy to offer students in Phase III of the Aberdeen undergraduate medical curriculum on which we now report.

Phase III of the undergraduate curriculum is the student's clinical attachment phase where they receive their core clinical knowledge training. Students rotate through nine clinical attachments, each of 5 weeks, with each attachment broken down into smaller units and taught in a systems-based approach. We based our initial development strategy on the 'Breast' teaching where students attend the Breast Unit forming 1 week of the 5-week Reproductive/Breast/Genito-Urinary Medicine attachment.

Development of the anatomy module (supplemented with other basic science material) was dependent on an initial discussion with the appropriate clinical lead and consultant colleagues on the basic 5–10 anatomical points that they would expect a student to understand whilst on their attachment. With this information, the prototype Breast learning module created had three components:
(1) An introductory formative quiz – true/false questions to quickly identify areas of strength or weakness in anatomical knowledge and understanding.
(2) A learning package, hard copy and electronic, to cover the areas of anatomy required for the Breast clinical attachment.
(3) A final assessment which could be capable of acting as a summative assessment.
This 'Breast' package has been used to assess both staff and student interest in developing learning packages to support clinical attachments. The main features of the prototype package will be illustrated.

Introducing anatomy learning packages into clinical attachments: the course co-ordinators' views

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In Aberdeen, we have resolved to address concern about the level of anatomical (and other basic science) knowledge gained by medical undergraduates and which is retained into the later clinical years of the undergraduate training programme. Teaching co-ordinators in Phase III, a clinical attachment phase (students rotate through all specialties in a series of nine 5-week blocks in a systems-based approach, with a co-ordinator for each block) of the Aberdeen medical curriculum, were interviewed concerning the need for learning packages that provided vertical integration of anatomy, and other 'basic' sciences, in association with their clinical attachment. The interviews were semi-structured and pre-arranged (with a broad outline of the nature of the study provided), and many of the co-ordinators had discussed the main issues with colleagues prior to being interviewed. Part of the interview concerned closed-style questions concerning the need for learning packages: it is the responses to these latter questions that are reported on here.

A total of 46 of 58 co-ordinators from two teaching centres (Aberdeen, Inverness) were interviewed; 28 were conducted 'face-to-face', 17 through email, and one by telephone, over the summer of 2007. All clinical disciplines were invited to participate and only two saw no relevance of the study to their clinical area (both Public Health).

Do you think there is a need to develop the teaching/learning of anatomy and basic science in your clinical field? – 91% said yes.

Would you be willing to help develop a teaching package for Phase III students? – 51% said yes; 42% said yes but I have no time to do this; 7% said no.

Do you think teaching/learning packages would add to the student workload unnecessarily? – 66% said no; 24% they should know their anatomy and basic science; 10% said yes.

How best should the teaching/learning packages be delivered? – 61% selected self-directed learning as the best method; 21% first day of the block; 18% on the ward.

There was, therefore, a clearly expressed view for anatomy and other basic science learning packages to support clinical attachments but less interest in participating in the development and delivery.

Poster presentations – D. Theme: Miscellaneous

Quantitative studies on the effects of prenatal exposure to nicotine on renal histology and gene expression in spontaneously hypertensive and Brown Norway rats

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In earlier studies, we have demonstrated that 9-week-old spontaneously hypertensive rats (SHR), but not age-matched normotensive Brown Norway (BN) rats, display a decrease in kidney weight and increase in blood pressure in response to prenatal exposure to nicotine (PEN), a main component in cigarettes. Here, we examined further the effects of PEN on morphology and genome-wide gene expression in the kidneys of the two strains. Nicotine bitartrate (6 mg kg^{-1} body weight) or saline bitartrate was administered to dams via subcutaneous osmotic minipumps throughout gestation. Kidneys were removed from SHR (nicotine: $n = 13$ and control: $n = 12$) and BN (nicotine: $n = 12$ and control: $n = 12$) offspring. Five kidneys per group were randomly selected to assess renal morphology at the light microscopic level using systematic random sampling and stereological analysis of renal composition and glomerular number and size. Total RNA and protein were extracted from kidneys. RNA samples from individual animals were pooled within each experimental group to perform microarray gene-expression analysis (Affymetrix®; Genespring, GX). Expression of genes of interest was subsequently verified with Western blotting. Data were analysed using two-way ANOVA with Strain and PEN as the main factors. SHR, but not BN, rats treated with nicotine vs. saline showed a trend towards decreased total kidney volume. No significant effect of PEN on cortical or glomerular volume or on glomerular number or size was observed in either strain. The microarray analysis showed a significant up-regulation of the angiotensin type 1 receptor b (Agtr1b) in SHR but not in BN rats treated with nicotine vs. control. A similar trend was observed in Agtr1 protein level (types 1a and 1b assessed together). Our results suggest that in SHR, but not in BN rats, PEN decreases overall kidney size without altering glomerular number or size and up-regulates intra-renal expression of the Agtr1b. The latter may be related to the effect of PEN on blood pressure observed previously only in SHR rats.

Differential parenchymal growth in the adrenal gland of the spontaneously hypertensive rat (*SHR/Ntac)* model of genetic hypertension

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The spontaneously hypertensive rat (*SHR*) provides a well-accepted animal model for the study of human essential hypertension. However, the factor(s) responsible for the genesis and maintenance of this hypertension remain controversial. Despite the well-recognized involvement of the adrenals in the development of hypertension in SHR, studies concerning the precise nature of adrenal structural alteration are not well documented. The present study used unbiased stereology to determine the possible changes in adrenal gland parenchyma in *SHR* at a time when hypertension has been well established. Twelve-week-old ($n = 12$) male *SHR* and a similar number of their age-matched normotensive counterparts (Wistar Kyoto, *WKY* rats) were used. At the age of 22 weeks, when hypertension is established in SHR, each animal was anaesthetized with intraperitoneal sodium pentobarbitone and perfusion-fixed with Karnovsky's fixative. The adrenal glands were removed and processed for light and electronmicroscopic stereology (Gundersen *J. Microsc*. **143**, 3–45, 1986; Mayhew and Gundersen *J. Anat.* **188**, 1–15, 1996). All data were presented as mean ± SEM. Differences between the *SHR*/*Ntac* and *WKY* were assessed using 2-factor ANOVA. Numerical density of glandular epithelia in the cortex had increased significantly whereas, in the medulla, it had decreased. ($P < 0.01$). The volume-weighted mean volume of nuclei was unchanged in the cortex but significantly decreased in the medulla in SHR as compared with WKY. However, the volume-weighted mean volume of cells in the cortex had increased by about 7% ($P < 0.05$) and in the medulla by 13% ($P < 0.01$) in SHR as compared with WKY. There were no significant differences in the number-weighted mean volumes of nuclei or cells in the cortex or medulla in SHR as compared with WKY. Changes in volume density of nucleus and cytoplasm in cells of the cortex and medulla between animal groups were not significant. This study has demonstrated that in SHR there exists a significant cortical hyperplasia of the adrenal gland parenchyma. This might well be related to the genesis of genetic hypertension in this strain of animals.

Histological investigation of the nature and incidence of osteoarthritis in the knee joint with focus on the contribution of the tidemark

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The pathophysiology of osteoarthritis (OA) is elusive with much debate on the contribution of the components of a diarthrodial joint. The aim of the study was to further elucidate the pattern of osteoarthritic change in the knee joint. Eleven embalmed cadavers (four males and seven females) were selected at random from the Dissection Room at King's College London and the tibial plateau was prepared for histological examination. The OARSI (OA Research Society International) OA cartilage histopathology assessment

grading system was applied to measure the incidence and severity of OA within these samples (on a scale of 0–6). All of the cadavers showed signs of OA with the degree of degeneration varying across the surface of the joint, indicating that it is responsive to localizsed biomechanical forces. The data show that the process is dynamic and that there is a hypertrophic response of the articular cartilage resulting in a maximal thickness at grade 3 but a progressive decrease thereafter until complete denudation of the cartilage. The response is initially mirrored in the subchondral plate but at grade 3 there is considerable thinning with recovery of plate thickness at higher grades. Examination of the sections showed that there is an intimate relationship between the tidemark (a calcification front that defines the zone of calcified cartilage from articular cartilage) and chondrocytes, which could be as a result of tidemark progression into the cartilage. Progression provides an explanation for cartilage thinning and subchondral plate thickening in later stages of OA, facilitating joint surface support in mechanical loading. Subchondral bone was observed to be in close apposition to the tidemark in most sections, resulting in a loss of calcified cartilage in these areas. The combination of calcified cartilage remodelling to subchondral bone and a disproportionate progression of the tidemark could contribute to subchondral plate thinning at grade 3. The data highlight that OA is a disease of the entire diathrodial joint and reveal a much more active role of the tidemark in OA pathophysiology than previously suggested.

Can cranial neural crest-derived cells help repair cartilage? A continuing story

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Osteoarthritis (OA) has recently been seen with greater frequency in the western world due to the aging population in these areas. At present one of the most common treatments for OA is total knee replacement. However, this surgical procedure is expensive and time consuming and is possibly unsustainable if the current population trend continues. This has led many groups to look for another way to treat OA. The possibility of using multipotent cells in treatments is an exciting possibility that has so far met with difficulties, including the inability of these cells to integrate and produce fully functioning cartilage. Cranial neural crest cells (CNCC) have been largely overlooked, yet they have the great ability to migrate and integrate into various tissues and are also known to give rise to cartilage. We propose to study the potential of this group of cells to give rise to cartilage when injected into a new environment such as the developing limb. We have identified the region of the developing limb that gives rise to the femorotibial joint and the intertarsal joint in the chick to establish the area into which CNCC will be injected. We have subsequently been injecting chick CNCC that have been removed from the mid/hindbrain region and labelled with Lac Z, into developing limbs of chicks at HH stages 17–18 and have begun analysing the results using histological techniques, including staining using Eosin or Alcian Blue. This study has demonstrated that cranial neural crest-derived cells (CNCDC) retain their ability to give rise to cartilage when implanted into the developing limb. The Lac Z labelled CNCDC can be seen in many structures throughout the length of the limb. Whether these cells can be manipulated to give rise solely to cartilage within the joint regions is an exciting question we hope to answer. These results are very encouraging as we may be able to learn more about whether CNCDC can integrate into damaged cartilage as well as developing cartilage.

Localization of sulfate anion transporter sat-1 in muscular tissues

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Sat-1 is a sulfate and oxalate transporter which has been localized and characterized in kidney and liver tissue at both mRNA and protein levels. Using a *Xenopus laevis* expression system, we found that [^{35}S]sulfate and [^{14}C]oxalate uptakes in sat-1-expressing oocytes at saturating sulfate and oxalate concentrations were 33.4 ± 7.8 and 20.1 ± 5.9 pmol/5 min·oocyte, respectively, indicating slight preference for sulfate [K_m:162 ± 26 μM (sulfate); 53 ± 5 μM (oxalate)]. Gene expression for sat-1 has been studied in various tissues. Clear expression was found in murine heart as well as murine and rat skeletal muscle. No expression was detected in rat heart or human rat and skeletal muscle. In contrast, its presence at the protein level in muscular tissues has not been investigated to date. Here, we studied sat-1 distribution in rat heart, soleus (rich in slow fibres) and extensor digitorum longus (rich in fast fibres) muscles immunohistochemically. Tissue was taken from male Sprague Dawley rats (4–6 months old). Samples were fixed in 4% phosphate-buffered paraformaldehyde by immersion, cryoprotected, frozen and cryosectioned. Sections were examined by means of routine histological staining or were stained immunohistochemically to detect sat-1. Negative controls were run parallel to each immunostaining by omitting the primary antibody. Preliminary findings on tissues of two rats showed that sat-1 can be visualized associated with the plasmalemma of the skeletal muscle fibres in both muscles. Additional intracellular fine trabecular mesh-like staining could also be observed. This was more obvious in the extensor digitorum longus. Skeletal muscle staining was not constantly seen and often had a discontinuous appearance. The pattern observed in skeletal muscle was not seen on myocardiocytes. These only showed sporadically fine intracellular signal. Staining was also occasionally seen in endothelial and connective tissue cells and was rare or absent in arteriolar smooth muscle. These results suggest that sat-1 can be detected associated to the cell membrane of skeletal muscle fibres and that it seems to be more relevant for skeletal muscle than for heart tissue, in which no significant localization was found. The fact that, in the skeletal muscle fibres, staining was not consistently seen may speak for low amounts and/or low availability on skeletal muscle cells for immunohistochemical demonstration.

Lectin staining for evaluation of angiogenesis in enzymically cross-linked collagen scaffolds used as dermal substitutes in rats

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Collagen is commonly used in dermal tissue engineering but the use of this biomaterial is not without drawbacks. *In vivo*, the rate of degradation of collagen-based scaffolds can be greater than the rate of dermal regeneration. The challenge is to maintain structural integrity of the scaffold until healing is complete without compromising its cytocompatibility. Microbial transglutaminases are transamidating enzymes that introduce covalent bonds into protein structures and, in particular, they catalyse the cross-linking of glutamine and lysine residues in collagen molecules. We hypothesized that the structural integrity of collagen scaffolds cross-linked with mTGases would be maintained until healing is complete, while retaining a cytocompatible environment. Freeze-dried untreated and mTGase cross-linked collagen type 1 scaffolds were fabricated and applied to full thickness 1-cm^2 wounds in the dorsum of male Sprague Dawley rats (n = 6). In a previous study, angiogenesis was evaluated in specimens stained with Masson's trichrome. Here, we report the use of a biotinylated lectin [isolectin B_4 (BSI-B_4) of *Bandeiraea simplicifolia*] as a more specific marker for the detection of blood vessels. The use of this lectin is a novel approach to the detection of angiogenesis in dermal substitutes. Stereological methods were used to quantify blood vessels at three time points (7, 14 and 21 days) after implantation of the scaffolds. We then compared these data to those previously obtained using Masson's trichrome. Both methods demonstrate that there is no statistical difference in the surface density of new blood vessels in untreated collagen scaffold and in collagen scaffolds that were cross-linked with mTGases.

The effect of lymphocytes on Caco-2 intestinal epithelial permeability

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Earlier communications have demonstrated increased intestinal epithelial permeability and particle uptake when a Caco-2 model was made more like the *in vivo* situation by exposure to sub-epithelial macrophages (at a ratio of 1 : 1). New data have now been collected showing the effects of Raji B-lymphocytes, either below the epithelial membrane (sub-epithelial lymphocyte model) or cultured with it (intra-epithelial lymphocyte model). Caco-2 cells were cultured at 37 °C for 21 days on 3-μm porous polyester membranes. Lymphocytes (sub-epithelial) were added to the lower well of Caco-2 cultures (1 : 10) on day 20 and incubated together for 24 h as for the sub-epithelial macrophage model. Lymphocytes (intra-epithelial) were also seeded on the membrane with Caco-2 cells (1 : 10) on day 0. On day 21 of culture, all groups were exposed apically to 2-μm latex particles for 5 or 60 min. Epithelial permeability was measured using transepithelial resistance (TER), with a decrease in TER suggesting a tight junction (TJ) loosening: sub-epithelial particles were also counted. Sub-epithelial and intra-epithelial lymphocyte models had a significantly lower TER_{day21} than Caco-2 cells alone, implying more permeable epithelia, and a reduced TJ closure 5 min after particle addition: 60 min after particle exposure the TER for the sub-epithelial lymphocyte model was higher than that for the intra-epithelial but not the Caco-2 model. The overall effect on TER, for both lymphocyte models before and after particle addition, was therefore similar to that caused by the presence of macrophages, but occurred to a lesser extent. However, only the intra-epithelial lymphocyte model showed increased particle uptake, to a similar level as the sub-epithelial macrophage model and at both time points. In summary, these preliminary data showed that the use of a Caco-2 model without the addition of *in vivo* relevant cell types might underestimate the rate of particle uptake and supported the finding from an *in vivo in situ* model that particle uptake was substantial at villous epithelium containing intra-epithelial lymphocytes.

Role of CLIP-170 in microtubule organization in cultured and *in situ* epithelial cells

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In most undifferentiated cells, microtubules are arranged in a classic radial array focused on the centrosome. During epithelial differentiation, the microtubule cytoskeleton is reorganized into a non-centrosomal-anchored apico-basal array. Recent evidence from our laboratory suggests that microtubule plus-end cortical interaction is a vital intermediate step in the formation of these apico-basal arrays and that the microtubule plus-end associated protein CLIP-170 is emerging as a key player. In polarized epithelial cells, CLIP-170 localized not only to the plus-end of microtubules but also to cortical sites and this association is microtubule independent. Depletion studies of CLIP-170 using siRNA in cultured confluent retinal pigment epithelial (ARPE-19) cells revealed delayed aster formation and the assembly of fewer microtubules following nocodazole removal. Later stages of nocodazole recovery revealed compromised cortical capture of microtubules and the generation of disorganized arrays which lacked centrosomal focus. IQGAP1, a Rho GTPase effector, is a potential cortical binding partner for CLIP-170 and preliminary data suggest that CLIP-170 and IQGAP1 co-localize at cortical junction-associated sites. siRNA depletion of IQGAP1 resulted in the loss of microtubule cortical interactions and the assembly of unfocused disorganized microtubule arrays. Data on microtubule organization in cochlear epithelial cells from double CLIP-170/CLIP-115 knockout mice will also be presented.

Cell density and 3D digital analysis confirm colocalization of both FSH and LH with GH in human embryo-fetal pituitary cells

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Pituitary cell lineages are thought to be defined from about the 6th week of embryonic development (Puy & Asa, 1996). We have previously reported colocalization of FSH and GH in human fetal pituitaries at 15–20 weeks and that clusters of such colocalizing cells occur in particular patterns. We have now extended the study to 8–10 weeks (the embryonic/fetal border) using the same antibodies in various combinations. Colocalization of FSH and GH was demonstrated at these earlier stages and differed only in detail from that seen in older fetuses. Colocalization of LH and GH was also detected, but to a lesser extent than that found for FSH and

GH, although the LH immunoreactivity appeared stronger than that for either FSH or GH. Cells colocalizing FSH-GH and LH-GH were usually close to a surface of the pituitary and to the peripheral blood vessels. Quantitation suggests that colocalization in 8–10-week tissue varies in both intensity and number of cells positive for FSH, LH, and GH. 3D digital analysis using Leica Confocal Software confirms both the colocalization and the more intense fluorescence in cells colocalizing LH and GH, which were not apparent in fetuses of 15–22 weeks. High magnification of colocalizing cells showed distinct immunoreactive spots for the gonadotrophins and GH, suggesting that subcellular colocalization was rarely complete. In standard confocal screen areas centred on groups of colocalizing cells, the cell density varied at 8, 9 and 10 weeks, respectively, for GH (3.2 ± 0.7, 4.3 ± 1.4, 5.5 ± 1.2), FSH (2.8 ± 1.1, 2.1 ± 0.6, 1.4 ± 0.6) and LH (2.8 ± 0.9, 1.9 ± 0.7, 1.3 ± 0.9). Colocalization with GH was detected in about 15–20% of the gonadotrophin-immunoreactive cells and may be slightly greater in the 9-week fetus. It certainly appears more common at these earlier stages than at later stages that we have examined.

The role of the *Dapper* molecules in paraxial mesoderm formation and muscle stem cell development in the avian embryo

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Muscular dystrophies is the collective name for a group of disorders, all characterized by progressive muscle wasting. As yet, no treatment is available. However, it has been suggested that the answer to this may lie in stem cell-based therapies. Stem cells are able to self-renew or differentiate, thus allowing them to maintain or regenerate tissues and organs. The understanding of the mechanisms regulating myogenic stem cells is crucial for the development of therapies for muscular dystrophies.

In all vertebrates, the entire skeletal musculature of the body plus the associated muscle stem cells develop from the segmented, paraxial mesoderm known as somites. Somite and muscle stem cell development require cell–cell communication that in part is mediated by Wnt signalling. However, the underlying molecular mechanisms are still unclear.

We have identified two novel multi-adaptor molecules that have been suggested to participate in Wnt signalling, namely Dapper1 and Dapper2. To investigate whether these molecules may be involved in muscle stem cell formation, we began to study their expression, regulation and biological function, using the chicken as model organism.

Dapper expression was analysed using whole mount *in situ* hybridization and compared to the distribution of a Wnt-dependent lacZ reporter. The regulation of Dapper expression was investigated, treating embryos with candidate regulatory molecules. The function of Dapper molecules was addressed, using RNAi-based knock down strategies.

Dapper molecules are expressed at sites of Wnt signalling. Dapper1 is expressed in the naïve paraxial mesoderm prior to somite formation. Dapper2, in contrast, is expressed in muscle stem cells in the somite. This suggests that the two Dappers mark distinct steps in mesoderm maturation/muscle stem cell development.

Expression of Dapper1 was found upregulated when embryos were treated with Fgf8, a molecule known to keep cells in an immature, undifferentiated state. Retinoic acid that initiates differentiation of many cell types was found to downregulate Dapper1. In contrast, expression of Dapper2 was downregulated by Fgf8, supporting the idea that Dapper1 is associated with naïve mesoderm and Dapper2 with a more committed state of myogenic cells.

Finally, when Dapper2 was knocked down, markers for muscle stem cells were downregulated, whereas genes driving muscle differentiation were upregulated. Thus, our preliminary data suggest that Dapper2 may be required to maintain myogenic stem cells in a precursor/stem cell state.

Education Symposium

Introduction to core skills in anatomy teaching

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Professionalism in the dissecting room

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The teaching and cultivation of professionalism is an integral part of medical education and central to maintaining the public's trust.

A tutorial with discussions on The Medical School Charter on student fitness to practise and Professional attributes was followed by a tutorial on examples of unprofessional behaviour in the Dissection Room (DR).

An anonymous questionnaire survey has shown the students to value the examples of un/professionalism in the DR, making it more practical and relevant. Several cases of unprofessional behaviour witnessed were recorded, although further discussion may be needed to differentiate between unprofessional and amateurish, but acceptable, behaviour.

The knowledge and skills to develop the values and attitudes of the medical profession should be systematically incorporated into the medical curriculum as early on as possible. With this strategy, students are given the opportunity to reflect on their ability to identify the attributes and reflect on their own and their peers' ability to develop and practise these attributes.

The first year DR teaching can be utilized as an educational strategy to nurture the development of the attributes of professionalism. It offers an ideal environment for reflection on the learning experience, personal performance as well as peer assessment.

Learning the language of anatomy

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Every professional community has its own language, with the assimilation of this new vocabulary being a pre-requisite for meaningful participation. This is particularly true for the study of medicine. Terminology in anatomy, a fundamental subject in the

medical disciplines, is largely based on Latin or Greek, languages taught relatively infrequently in the schools of English-speaking countries today. As a result, students struggle to cope with the new and unfamiliar language.

This interactive workshop is designed to investigate how (and whether) we as educators use etymology in our teaching. It will also present some research into whether knowledge of the meaning of an anatomical term helps students recognize an anatomical structure, as well as whether identifying an associated English word assists them in deducing its meaning.

Do students of anatomy need to communicate with different audiences?

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Communication skills have never been more relevant than in today's global environment. The ever-increasing development and use of communication systems and devices such as the internet, e-mail and mobile phones require that a clear interchange of information takes place so that miscommunication does not occur. The ability to communicate with society is one of the key skills by which our students can help enhance knowledge and understanding of different subjects within the general population. Unfortunately, up until recently few subject areas have provided training for their students in the art of communicating with different audiences, especially a non-specialist one. Anatomists have a long history of communicating with the public and therefore are in a powerful position to provide students with a solid framework for developing core skills in communication. Despite the increasing burdens on medical, dental and science curricula it is still possible, if not essential, to highlight the need for being able to communicate with different groups within society. A number of interactive and innovative opportunities exist for developing effective and useful oral and written communications skills of students, including the creation of lay statements, information leaflets, mock radio and TV interviews and interactions within the classroom. Implementation of such experiences within our courses will ensure we are preparing students fully for the specific challenges that an interconnected community demands.